



The Effect of Ascorbic Acid Ingestion on the Risk of Calcium Oxalate Kidney Stone Formation.

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by

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Summary

This study was undertaken to investigate the effect of prolonged megadose ingestion of ascorbic acid on the risk of calcium oxalate kidney stone formation. Ten healthy male subjects (ages 20 to 30 years) participated in the study. Each was required to ingest a daily dose of 4 g ascorbic acid per day for a period of 5 days. Twenty-four hour urines were collected at various times prior to, during and after this protocol. During each 24 hour collection, aliquots were withdrawn and stored in the presence and absence of an EDTA preservative. Other aliquots were withdrawn for immediate ascorbate analyses.

Urine samples were analysed for sodium, potassium, magnesium, calcium, citrate, oxalate, urate, phosphate and creatinine. A new flow-injection method was designed, developed and tested for the analysis of urinary ascorbate. Analytical data obtained from this method were compared with results obtained from a manual titration method using 2,6 dichlorophenolindophenol. While the accuracy of both methods was found to be similar, results obtained by the flow-injection were more repeatable. All urine samples were analysed for ascorbate by both methods. *In vitro* crystallization experiments were performed on each urine to determine the limit of metastability and the rate of calcium oxalate crystallization. All data were statistically compared using the method of least-squares.

Prolonged ascorbic acid ingestion had no effect on urinary calcium and oxalate nor on several other biochemical risk factors including urinary citrate, sodium, chloride and creatinine. Calcium oxalate metastable limits and crystallization kinetics were also unaffected, as were

values of the Tiselius risk index and calcium oxalate relative supersaturation. While risk-reducing changes in urinary magnesium and pH occurred, these were offset by risk-increasing changes in urinary potassium and phosphate.

The investigation showed that *in vitro* conversion of ascorbate to oxalate occurs but that this can be prevented by the presence of EDTA in the collection vessel. The study also demonstrated that ingestion of large doses of ascorbic acid probably commences with high percentage absorption of the vitamin but that saturation is achieved within 24 hours. Continued ingestion results in less absorption with excess ascorbic acid being excreted unchanged in the urine.

In a separate case study, prior to the commencement of the investigation described above, a healthy male participant was given a daily dose of 8 g ascorbic acid in a protocol which was planned to last 9 days. Significant crystalluria and haematuria developed on the 8th day after which further ascorbic acid ingestion was immediately suspended. In this individual, several risk factors changed significantly. These were urinary oxalate excretion, metastable limit, Tiselius risk index and calcium oxalate relative supersaturation.

This study shows that prolonged ingestion of large doses of ascorbic acid does not increase the risk of calcium oxalate kidney stone formation. However, the results of the special case study highlight the fact that in some individuals, renal handling of ascorbic acid may be impaired, leading to undesirable consequences.

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Chapter 1.

Introduction

Kidney stone disease is diverse involving consideration of many factors other than ascorbic acid overindulgence. This has led to a great deal of consensus and controversy in the study of urolithiasis. This introductory chapter covers a broad spectrum of aspects ranging from a comprehensive history of ascorbic acid, more commonly known as vitamin C, to an overview of urolithiasis relating and linking the risk factors and stone forming mechanisms of urine. Oxalic acid, the biochemical end product of the metabolism of ascorbic acid, is discussed. The existing literature perspective of the effect of ascorbic acid on the stone forming potential of urine is detailed.

1.1 Ascorbic Acid in General

Ascorbic acid plays a role in health and disease which has given rise to numerous controversial issues. A deficiency in the vitamin is sufficient to emphasise its essential need in order that the unpleasant symptoms of scurvy be avoided. However, controversial issues as to the effect of the vitamin in the prevention of common colds and influenza, together with the role it plays in the treatment of cancer, are causes of concern to the medical fraternity. This is because one of the end products of ascorbic acid's biochemical metabolism is oxalic acid, which is a possible causative factor in urolithiasis.

1.1.1 History of Ascorbic Acid

Scurvy is defined as a disease which produces haemorrhaging into tissues, bleeding gums, loose teeth, anaemia and general weakness. It is a deficiency disease which must have existed throughout history, but was particularly noted during the long sea voyages of the Middle Ages. It would appear that there was a lack of communication in ancient medicine

since documentation of ascorbic acid as a cure for scurvy dates back as far as the 13th century [1] and further reports do exist in the following four centuries.

It has been known for a long time that the anti-scorbutic effects of fresh fruit and vegetables diminish after the time of harvest. This occurs because of the loss of ascorbic acid due to a variety of oxidative processes involving oxygen from the air. This contributed to the confusion over the efficacy of fresh fruit and vegetables in the prevention and cure of scurvy, even as late as the beginning of the 20th century.

Scurvy resulted in the death of many a sailor in the 16th century, since fresh fruit and vegetables were unavailable on the long sea voyages of the period. Eventually it was recognised that fruit juices diminished the number of cases of scurvy aboard the ships. The journal of Sir James Lancaster's voyage in 1601 shows the essential value of oranges and lemons with the quote [1],

"... the reasons why the general's men stood better in health was this; he brought to sea with him, bottles of the juice of lemons, which he gave to each one, as long as it would last, three spoonfuls every morning ... by this means the general cured many of his men ..."

With hindsight, it is easy to recognise the necessity to establish the effectiveness of fresh fruit and vegetables in the cure of scurvy in order to overcome the confusion of the treatment of the disease. James Lind was the first to embark on a "clinical trial", of the various treatments of scurvy.

The trial, shown in Table 1.1, consisted of twelve men with scurvy being maintained on the same daily diet. Six treatments were applied in groups of two and it was found that the men on the citrus fruit treatment showed sufficient recovery to resume normal duties after two or three days. The other treatments had little or no effect on the disease, with the result that Lind concluded that citrus fruit was the most effective remedy for scurvy. However, Lind's recommendation that green vegetables, oranges and lemons be carried aboard on long sea voyages went unheeded. It appeared that he did not have sufficient influence in this regard and it was only when Sir Gilbert Blane was appointed as physician to the Household of the Prince

of Wales that the advice was heeded. In 1795, Blane became a commissioner to the Board of the British Navy and he authorised that lemon juice be a standard issue to the British Navy. The incidence of scurvy dropped dramatically and it is considered that it is largely due to Lind and Blane that this disease has been conquered at sea [1].

Table 1.1 *A summary of James Lind's trials to find possible cures for scurvy.*

| Group | Treatment |
|-------|---|
| 1 | One quart of cider |
| 2 | 25 drops of elixir of vitriol three times a day |
| 3 | Two spoonfuls of vinegar before meals |
| 4 | Half a pint of seawater |
| 5 | Two oranges and one lemon |
| 6 | A medicinal paste containing substances such as garlic and mustard seed |

Scurvy does occur on land, particularly in regions where fresh fruit and vegetables are unavailable during the winter months. This disease was more prevalent in the aristocracy since vegetables were considered to be a food for the lower class because they grew in "dirty soil". Much of the failure to conquer scurvy, even at the beginning of this century, is due to a continuing reluctance, despite evidence, to recognise it as a deficiency disease. Arguments as to the cause of the disease continued well into the first quarter of the 20th century. It was only after the discovery of foodstuffs which showed anti-scorbutic properties, that arguments diminished. Anti-scorbutic properties in certain products were identified, isolated and shown to be effective in the cure of scurvy. The first 25 years of this century resulted in the scientists unravelling the mystery of the elusive anti-scorbutic factor.

Holst and Frolich (1907) [cited by 1] used guinea pigs to test various dietary effects in attempts to ascertain the extent of anti-ascorbic activity. They were able to show the effectiveness of fresh fruit and vegetables conclusively. In 1912, Casimer Funk showed that scurvy, together with various other diseases, is a deficiency disease which can be prevented in the presence of small amounts of nitrogen. He proposed that all these compounds were amines, and referred to them as “vital amines” which has since been abbreviated to “vitamines”, and then to the more commonly known “vitamins” [1].

1.1.2 Discovery and Structure

The First World War led to further experimentation by a groups of women at the Lister Institute, which led to the development of a bioassay for ascorbic properties in various food stuffs. The decade from 1920 to 1930 showed extensive research in the investigation of ascorbic acid, so called after an attempt to name the anti-ascorbic factor, water soluble C. The Lister Institute established the reducing properties of the compound, despite the fact that the compound could not be isolated. This property was then used in chemical analysis of the compound, which avoided the time consuming bioassays previously employed.

Numerous research groups tried to isolate the elusive chemical compound, but despite the attempts of these groups the stage was set for an unknown Hungarian scientist, Albert Szent-Gyorgyi. Szent-Gyorgyi isolated a substance from the adrenal cortex of cattle (procedure detailed in [1]) and found that the substance, like all reducing agents, decolourised iodine. He calculated the relative molecular mass to be 88.2 or a multiple thereof. The lowering of the vapour pressure of water suggested a relative molecular mass of approximately 180, indicating 176.2 to be the correct value. Finally, combustion analysis enabled the molecular formula to be established as $C_6H_8O_6$. The compound was published under the name *hexuronic acid*. Despite the fact that Szent-Gyorgyi thought that hexuronic acid was ascorbic acid, his dislike of clinical trials prevented further work on the compound. Eventually the true nature of hexuronic acid was identified in 1932 by Svirbely, one of Szent-Gyorgyi's students. Szent-Gyorgyi then discovered that the vitamin was present in Hungarian paprika and that

isolation from this plant was simple; as a result, sufficient amounts of the compound could be isolated and its chemical structure determined [1].

Structure elucidation was a long and involved process carried out by Edmund Hirst. Boiling with hydrochloric acid indicated that at least 5 of the 6 carbons formed an unbranched chain. Ascorbic acid was then determined to be a weak monobasic acid and strong reducing agent. The first step in oxidation was reversible and involved the loss of two hydrogens, the product being named dehydroascorbic acid. The reversible oxidation with iodine was analogous to the known reaction with 2,3-dihydroxymaleic acid, indicating the possible presence of an ene-diol grouping, $C(OH)=C(OH)$. Despite the acidity of the ascorbic acid, a carboxylic group was not found to be present. Dehydroascorbic acid was shown to be a neutral lactone, and with the easy inter-conversion between the two compounds, the possibility of ascorbic acid also being a lactone was indicated. It was also known that two more alcoholic OH groups were present. Further reaction resulted in the formation of a derivative which still contained the two enolic hydroxyls. Further oxidation of the dehydroascorbic derivative yielded oxalic acid and threonic acid. This established the stereochemical relationship of natural ascorbic acid as belonging to the L-series of sugars and also confirmed that the lactone carbonyl was directly adjacent to the ene-diol grouping. A series of organic reactions was carried out on the compound in an attempt to establish the size of the lactone ring. Ascorbic acid was shown to be a γ -lactone (Figure 1.1).

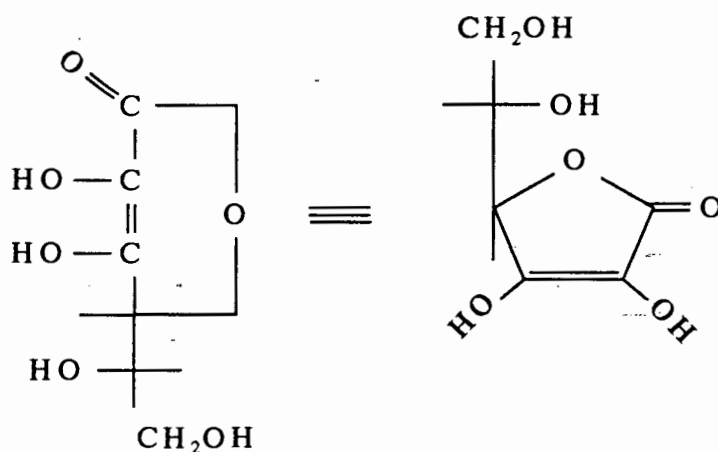


Figure 1.1 *Fischer projection formula (left) and structural formula (right) of L-ascorbic acid [1].*

The Birmingham group confirmed the structure of ascorbic acid by developing a synthetic route to L-ascorbic acid. The physical properties of L-ascorbic acid have been summarised in Table 1.2.

Table 1.2 *A summary of the physical properties of L-ascorbic acid [1].*

| | |
|--|--|
| Nomenclature | Hexuronic acid, cevitamic acid, redoxon |
| | L-ascorbic acid, L-xylo-ascorbic acid |
| | L-threo-2,3,4,5,6-pentahydroxyhex-2-eonic acid-4-lactone |
| | L-threo-hexono-1,4-lactono-2-ene |
| Melting Point (°C) | 190 - 192 °C with decomposition |
| Relative Molecular Mass | 176.14 |
| pK _a | first (C-3-OH) = 4.25 |
| | second (C-2-OH) = 11.79 |
| Specific Rotation | $[\alpha]_D^{20} = + 23^\circ$ in water |
| Solubility (g.100 cm ⁻³ @ 20° C) | 33(water), 3(ethanol), 1(glycerol), insoluble in benzene, ether, chloroform, fats and oils |
| Density | 1.65 g.cm ⁻³ |

The work, published in 1933, was the culmination of painstaking and laborious synthetic chemistry; as such, it was appropriate that the Nobel Prize was awarded to Szent-Gyorgyi in 1937. The growth and development of spectroscopic techniques in the years following the successful elucidation of the L-ascorbic acid structure have enabled it to be examined more closely. Further instrumental analysis of the structure of ascorbic acid included X-ray

diffraction, ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic spectroscopy and mass spectrometry [1].

1.1.3 Medical Aspects

The exact physiological role of ascorbic acid is not fully understood, despite its numerous successful applications in various unrelated medical situations. Ascorbic acid has been used in the treatment of scurvy for hundreds of years. It has also been used in other medical conditions, with the result that megadose quantities are consumed daily. This is far in excess of the relatively tiny amount needed to prevent scurvy. Ingestion of quantities which significantly exceed the recommended daily diet are a cause of some concern since these excessively high intakes may cause undesirable side effects such as diarrhoea [2] and the possible increase in the kidney stone forming potential of the urine [1].

There are a number of diseases other than scurvy which have been associated with ascorbic acid deficiency, including anaemia, hypochondriasis and depression. Although it is widely believed that scurvy has been eliminated as one of the deficiency diseases in the western world, it still exists in the chronically ill, the institutionalised elderly, chronic alcoholics and various other groups. For a healthy individual, 10 mg daily of the vitamin will prevent the onset of this disease, which seems to manifest when the body pool of ascorbic acid drops below 300 mg [1].

Increasing daily ascorbic acid dosage to approach megadose proportions is of little benefit, since absorption of the vitamin is limited. Approximately 95 % of a 100 mg daily dose is absorbed, whereas only 20 % of a 5 g daily dose is absorbed [1]. The low renal threshold ensures that excess plasma levels are excreted and that there is a rapid increase in liver ascorbate catabolizing enzymes. When the plasma pool is between 1.4 and 2.0 %, the tissues are probably saturated with a body pool of up to 3000 mg [1]. Any excess dietary ascorbic acid is then excreted in the urine as ascorbic acid or one of its oxidative products such as oxalic acid. If there is a deficiency the tissues will absorb the ingested ascorbic acid; however once the tissues are saturated or if they are saturated at the outset of ascorbic acid ingestion, most of the ascorbic acid is excreted during the next 24 hours [1].

In addition to the prevention and treatment of scurvy, ascorbic acid is used therapeutically in numerous other situations such as surgery, physical trauma and duodenal ulcer treatment since the vitamin is known to assist in the healing of wounds as well as to aid the body in fighting and preventing infection. Ascorbic acid is also used in cases of diarrhoea, fever and in instances where the risk of inflammation and infection is high. Ascorbic acid can also be used in conjunction with iron supplementation in cases of anaemia due to a deficiency of iron. Controversy surrounds the use of ascorbic acid in a number of medical conditions, ranging from the common cold to mental illness, infertility and cancer [1].

1.1.4 Toxic Effects

Since Linus Pauling's suggestion that ascorbic acid may decrease the chances of cancer [cited by 1], many conflicting opinions have arisen in the literature, with the suggestion that ascorbic acid may even cause cancer. Evidence in this respect is mainly based on animal experimentation and is difficult to extrapolate to the human situation. Further concern, and of implicit relevance in this study, is that the conversion of ascorbic acid to oxalic acid results in an increase of oxalate in the body and hence increases the risk of calcium oxalate kidney stone formation. Large doses could also have the effect of slight discomfort of the intestinal tract, resulting in diarrhoea. Ascorbic acid may also be administered to enhance the effect of drugs that increase urinary output in patients with iron-overload. This is of some concern since the vitamin may actually increase the toxicity of iron which may lead to myocardiopathy in some patients.

1.2 Oxalic Acid

Since calcium oxalate concretions are the most common type of kidney stone [3], a discussion of the chemistry and the properties of oxalic acid is of importance in defining the pathogenesis of calcium oxalate nephrolithiasis [4]. Furthermore, since an understanding of the mechanisms underlying the formation of calcium oxalate stones is essential, much interest in the metabolism and excretion of urinary oxalate exists [5]. While calcium metabolism and

excretion have been investigated at length with regard to its relationship in nephrolithiasis, it is only in recent years that more attention has been focused on oxalic acid.

1.2.1 *Physiochemical Aspects*

Oxalic acid is the simplest carboxylic acid found in nature [3]. It is a significant constituent of many leafy green vegetables and plants and is also extensively used in industry as a cleaning agent. It is a strong organic acid which forms acidic and neutral esters and diesters and mono- and diamides. The principle significance of oxalic acid in man is the precipitation of the calcium salt, which is extremely insoluble. The solubility is little affected by a change in pH in the range found in urine [6]. Ryall *et al.* [7] indicate that even though controversy surrounds urinary pH of stone formers as opposed to normal control subjects, pH does in fact have an effect on the solubility of calcium oxalate crystals. Since calcium oxalate is insoluble, urine is often supersaturated with crystals of this salt [8]. There is much evidence to suggest that calcium oxalate crystallization is retarded in normal urine by a wide range of inhibitors [7, 9].

1.2.2 *Dietary Sources and Absorption*

Oxalic acid is found in a variety of foodstuffs. Some oxalate rich substances have been summarised in Table 1.3 [5]. It is difficult to estimate the average dietary intake of oxalate, primarily because of the problem of complete extraction of oxalate from foods in the experimental assays [3]. This is emphasised in a practical manner when oxalate content in tea and coffee is examined. The amount of oxalic acid in tea and coffee will depend on the strength of the beverage. Past reports have indicated a wide range of oxalate intake, from 100 - 1000 mg in a 24 hour period [Zarembski *et al.* 1962, cited by 3].

Table 1.3 *A range of foodstuffs known to be rich in oxalate [5].*

| | | | |
|----------|------------|-----------|--------------|
| beans | chocolate | marmalade | strawberries |
| beetroot | cocoa | parsley | sesame seeds |
| carrots | coffee | rhubarb | tea |
| celery | grapefruit | spinach | |

Absorption of oxalic acid by the gastrointestinal tract is of interest due to hyperoxaluria and other gastrointestinal diseases [10]. In normal man, gastrointestinal absorption of oxalate is generally poor. A large proportion of the oxalate ingested exists as relatively insoluble calcium oxalate and, as a result, the crystals remain in the lumen of the gut. Dietary oxalate is known to contribute between 10 % [5] and 20 % of the urinary oxalate [11]. Research has indicated that during fasting, approximately 2.3 to 4.5 % of the administered oxalate load is absorbed [12]. However, the use of ^{14}C -oxalate has shown that approximately 12 % of the administered tracer load is absorbed in the fasting state [4].

Furthermore, various animal studies have shown that the absorption takes place over the whole tract, including the colon, and that the process is not a carrier-mediated process, but rather a non-energy requiring, non-saturable diffusion process [13] which is influenced by the concentration of free calcium in the intestinal lumen [11]. The mechanism of passive diffusion described by Binder [13] indicated that if the human intestine showed a similar mechanism of oxalate absorption as in animals, then the rate of oxalate absorption would be influenced by the concentration of oxalate in the luminal fluid (i.e. an increase in oxalate concentration in the fluid of the gut would result in increased absorption). Thus, it is probable that the moderately increased oxalate excretion in stone formers is due to a small increase in oxalate absorption, which in turn is due to the fall in the intraluminal concentration of calcium [11].

1.2.3 Excretion and Renal Clearance

Calcium oxalate is known to form a large percentage of upper urinary tract concrements. It is precipitated in urine which is supersaturated with respect to calcium oxalate. The solubility of this compound is very low, even in aqueous solution; about 7 mg dissolves in 1000 cm³ of distilled water [5]. The concentration in urine is always higher than this value but other urinary components contribute to its solubility. The presence of oxalate in the urine reduces its stability and it may thus be that even slight changes in oxalate concentration may result in serious effects on its crystallization properties.

Urinary oxalate is important for the following reasons :

- the majority of urinary concrements are composed of calcium oxalate [5, 14, 15],
- the most common type of crystalluria is calcium oxalate, and
- oxalate exerts a pronounced effect on the crystallization properties of urine.

Oxalate has been shown to have a more powerful effect on the degree of calcium oxalate supersaturation in urine than has calcium [16]. Despite the enormous potential effects of oxalate on urolithiasis, relatively few investigations have been carried out in an attempt to establish the role of oxalate in the pathogenesis of the disease [7]. A possible explanation may lie in the fact that urinary oxalate is notoriously difficult to measure. To further complicate oxalate measurement in urine, ascorbic acid, if present, converts spontaneously to oxalate, particularly in alkaline conditions [17]. This must be taken into account when reviewing previous literature relating the oxalate excretion of stone forming individuals and normal controls. Robertson *et al.* [18] state that “the inability to measure urinary oxalate accurately and precisely undoubtedly accounts for many of the apparent discrepancies between those investigators who *have* found significant differences in urinary oxalate amongst idiopathic stone formers and those who have been unable to do so”. A literature search reveals several studies which report significantly higher oxalate excretion in stone formers than in normal individuals [5, 18, 19, 20, 21]. However, there are numerous papers which indicate the contrary [22, 23, 24]. A contributory reason for this dichotomy of opinion could be due to the nature of the wide daily fluctuations observed in many of the chemical components of the

urine [7]. As mentioned earlier, in addition to the already technically difficult assay, is the complication of ascorbate interference. As a result, investigations which did not take preservative precautions in order to prevent this conversion or which employed oxalate assays which promote conversion, should be regarded with caution.

Average oxalate excretion at night is generally found to be lower than average oxalate excretion during the day [25]. There are several factors which may cause an increase in oxalate excretion during the day. These include an increase in dietary oxalate during the day, the intake of oxalate precursors such as ascorbic acid, an increase in metabolic activity and a higher urine flow [5].

Urinary oxalate is derived from endogenous synthesis and absorption of oxalate in the gastrointestinal tract. Oxalate is a metabolic end product in man which cannot be further degenerated. As such, it is excreted virtually unchanged in the urine [3]. The metabolic pathways producing oxalate are shown in Figure 1.2.

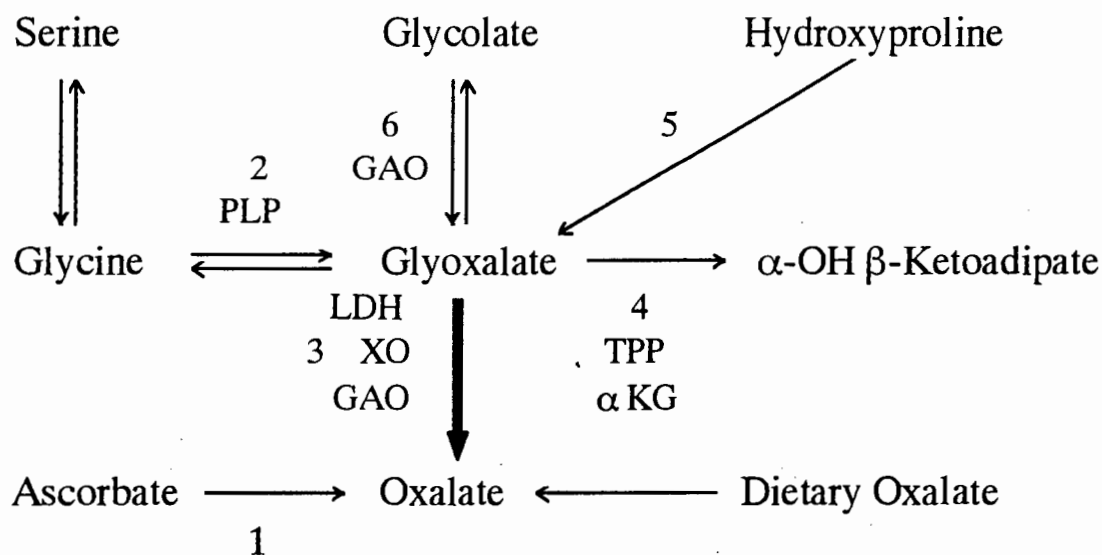


Figure 1.2 The known metabolic pathways in formation of oxalate in humans [5]. See text for details.

The two major sources of oxalate in man are the conversion of ascorbate to oxalate and the metabolic pathways of glyoxylic acid [3]. Oxalate production is an intricate network of reactions. The pathways to the formation of oxalate are discussed below. The numbers in parentheses correspond to the relevant pathways in Figure 1.2.

The conversion of ascorbic acid to oxalate (1) is of particular interest, especially since many members of today's health conscious society ingest large amounts of ascorbic acid on a regular basis [5]. This will be discussed in detail in Section 1.3.

The inter-conversion of glyoxylate and glycine (2) has aroused great interest. Essentially this reaction is a transamination with the active form of vitamin B₆ as the co-factor [5]. Literature investigations indicate that vitamin B₆ may prevent calcium oxalate stone formation [26, 27]. However, Tiselius [5] provides conflicting statements on the nature and effect of vitamin B₆ and its active form, pyridoxyl phosphate. Glyoxylate is a very important precursor of oxalate and the series of metabolic reactions concerned with the conversions of this reactive substance are complex. Briefly, lactic dehydrogenase (LDH) and nicotinamide adenine dinucleotide oxidise glyoxylate to oxalate (3) in the cytosol [Sawaki *et al.* 1966 cited by 3]. Glycolic acid oxidase (GAO) and xanthine oxidase (XO), although both capable of catalysing the conversion of glyoxylate to oxalate (3), are documented to be not of significant importance [28]. A further interesting process is the conversion of glyoxylate to α -hydroxy- β -keto-adipate (4) by means of α -keto-glutarate (α -KG). The reaction requires both thiamine pyrophosphate (TPP) and Mg²⁺. It is the major degradation pathway for glyoxylate and hence a low activity in this process would probably increase the formation of oxalate [5]. The inter-conversion of glyoxylate and hydroxyproline (5) is also not significant and hydroxyproline does not appear to be an important precursor of glyoxylate or oxalate [29]. The oxidation of glycolate to glyoxylate (6) is driven by glycolic acid oxidase (GAO).

Renal clearance of oxalate has been studied by isotopic methods [30, 31]. Filtration of oxalate at the glomerulus seems to be complete in view of the absence of significant protein binding of oxalate in the plasma. An oxalate : creatinine clearance ratio greater than one indicates that active secretion of oxalate must occur at some stage in the renal tract [30].

Numerous attempts have been made to determine the amount of oxalate absorbed by the gut. Tiselius [5] summarises the data from previous research. The absorption appears to be in the range of 2 - 12 % and assuming an average intake of oxalate of about 1 - 2 mmol per day, the dietary contribution to urinary oxalate is of the order 0.02 - 0.24 mmol. Oxalate rich foods would clearly increase this (Table 1.3).

Oxalate is absorbed in the gut by two processes: passive diffusion and active transport. The addition of metabolic inhibitors does not affect these processes. Absorption of oxalate is determined by the constituents of the intestinal fluids, the most important being calcium [5]. A high concentration of calcium oxalate in the intestine coincides with a reduced oxalate absorption. There are several other substances which form complexes with oxalate such as magnesium, aluminium and cholestyramine.

Hyperoxaluria is the abnormally high excretion of oxalate in the urine [32]. There are several interrelated nutritional links to hyperoxaluria and calcium oxalate stone formation, other than ascorbic acid. These factors are listed in Table 1.4.

Table 1.4 *Nutritional links related to hyperoxaluria and calcium oxalate stone formation [33].*

| |
|---|
| High oxalate consumption |
| Low calcium intake |
| Low magnesium intake |
| Ingestion of the common food preservative, EDTA |
| Primary or secondary deficiency of vitamin B ₆ |
| High dietary tryptophan |
| Primary or secondary deficiency of vitamin B ₁ |
| High blood levels of vitamin D |
| High sucrose intake |
| Inadequate water consumption |

The major causes of hyperoxaluria can be classified as two key pathogenic mechanisms : firstly, increased endogenous production of oxalate [3] primarily from its immediate precursors which include ascorbate, glyoxalate and glycolate [32] and secondly, increased oxalate excretion secondary to increased uptake or gastrointestinal absorption of oxalate [3] (i.e. the exogenous production of oxalate [5]).

The causes of hyperoxaluria in their respective pathogenic mechanisms summarised by Tiselius [5] are listed in Table 1.5.

Table 1.5 *Possible causes of hyperoxaluria.*

| Endogenous Oxalate | Exogenous Oxalate |
|------------------------------------|---------------------------|
| Ascorbate in excess | Excessive dietary oxalate |
| Vitamin B ₆ deficiency | Enteric hyperoxaluria |
| Primary hyperoxaluria | |
| Intake of other oxalate precursors | |

1.3 Ascorbic Acid and Kidney Stone Disease

Urinary oxalate is essentially derived from 3 major sources : dietary oxalate, conversion of two-carbon metabolites from glycolaldehyde and from ascorbic acid [34]. It has been established that dietary oxalate contributes approximately 20 % of the urinary oxalate [11], 40 - 50 % by two-carbon metabolites [35] and 30 - 50 % by ascorbate [36, 37]. The proportions from any one source will depend on the intakes of other exogenous sources and the rate of endogenous production [38].

It is well established that L-ascorbic acid metabolises to oxalic acid in the manner shown in Figure 1.3 [38]. Despite the fact that this conversion is well known, the complete metabolic

processes are not fully understood. It has however been established that the carbon atoms involved in the formation of oxalic acid are carbon atoms one and two [36].

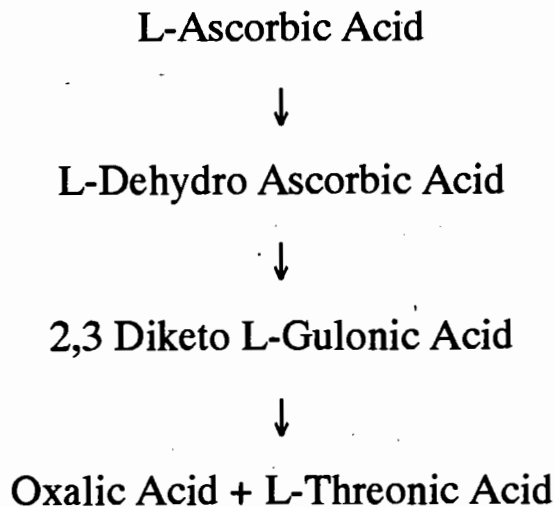


Figure 1.3 *The biochemical pathway of the conversion of ascorbic acid to oxalic acid.*

Absorption of ascorbic acid by the gut is an energy requiring process (Hornig *et al.* cited by 39]. The reabsorption mechanism for ascorbic acid appears to be limited to a maximum rate of $2.16 \text{ cm}^3 \cdot \text{min}^{-1}$; thus when the vitamin is presented to the tubules at a rate greater than this, the excess ascorbic acid is excreted in the urine [40]. The proportion of ascorbic acid excreted unmetabolised in the urine increases with increasing ascorbic acid intakes [39]. Thus, as the dose increases, the absorption decreases and the process eventually attains a level of saturation. It has been reported that large doses of ascorbic acid result in an excretion of approximately 80 % unchanged ascorbate in the urine [Takenouchi, 1966 cited by 41]. It has been suggested that given the efficient elimination by the kidney and limited absorption of ascorbic acid by the intestine, the chances of ascorbic acid overload are unlikely [Hornig *et al.* cited by 39].

Surveys indicate that 66 % of the general public uses vitamin and mineral supplements, either as multivitamins that include ascorbic acid, or alternatively, ascorbic acid on its own [33]. In today's health conscious society, ingestion of megadoses of ascorbic acid in excess of

the recommended daily allowance is common [42]. This is a cause of some concern considering that there is much controversy regarding the possibility that excessive dosage of ascorbic acid can increase intrarenal levels of oxalate to which the vitamin is metabolised, and hence that it can increase the risk of calcium oxalate lithogenesis.

Members of the medical fraternity have expressed concern about the long term use of ascorbic acid, and warn of its side effects [43, 44, 45]. Lamden [46] indicates that despite ascorbic acid being a highly valuable and useful substance, it is an exceptionally reactive compound biochemically and should thus be treated with respect. His concerns related to ascorbic acid ingestion do not only involve the elevated oxalate levels in the urine which may promote the possibility of kidney and bladder damage due to the formation of stones, but include various other medically related disorders. Briggs [44] reports a case study of a 21 year old male who after daily ingestion of 4 g of ascorbic acid showed an increase in urinary oxalate 11 times greater than when no ascorbic acid was being taken. Briggs remarks that the occurrence of individuals with high capacity to convert ascorbic acid to oxalate is probably rare, but unless they are identified, ascorbic acid supplementation at high doses could have undesirable results. There are numerous other papers which show that the ingestion of ascorbic acid increases the concentration of urinary oxalate significantly [25, 41, 42, 43, 47, 48]. On the contrary, practitioners who have been prescribing high daily dosage of ascorbic acid for both short and long periods do not report any toxicity [2, 49]. Other research studies have challenged the fact that oxalate levels are significantly raised, drawing attention to the various possible sources of error [38, 50, 51, 52, 53]. Singh *et al.* [54], show that ascorbic acid alone does not cause stone formation, though it may increase oxaluria in higher doses. A study by Hatch *et al.* [41] indicated that a significant increase in urinary oxalate occurred a week post cessation of ascorbic acid, rather than during the period of ingestion. This suggests that ascorbic acid may be retained and the effects delayed, possibly only manifesting after a dormant period.

L-ascorbic acid serves as a precursor of urinary oxalate in man, providing up to a third of the oxalate present in urine [36]. This pathway appears to have a low capacity since large amounts of ascorbic acid only cause moderate increases in urinary oxalate [55]. However, because the solubility of calcium oxalate in urine is low, even a small increase in urinary oxalate

in patients who are already hyperoxaluric could increase the likelihood of kidney stones [56]. Since the conversion of ascorbate to oxalate does occur, it might be expected that people with high ingestion of ascorbic acid may have high urinary oxalate. The literature however shows a conflicting picture, which can be divided into three sequences [38] :

- high intakes of ascorbic acid result in only a few cases of increased urinary oxalate;
- increase of urinary oxalate is moderate and occurs only in cases of extremely high ascorbic acid intake;
- large increases in urinary oxalate occur in most people on a high ascorbic acid load.

In an attempt to distinguish between these three groups, Hughes *et al.* [38] discovered a new pattern. Experimentation was carried out using four groups of subjects, each on different dietary loads of ascorbic acid. All subjects collected control urines (no ingestion of ascorbic acid) prior to ingestion of ascorbic acid loads of 1 g, 3 g, 6 g and 9 g per day. The results did not fall into any of the categories, but seemed to form a new pattern. Urinary oxalate concentration of each group was approximately doubled relative to control values, despite the different ascorbic acid loads. Thus, although there was an initial increase in urinary oxalate excretion, oxalate concentration in the urine did not increase with increasing ascorbic acid loads. None of the subjects showed a massive increase in urinary oxalate excretion, even after a load of 9 g of ascorbic acid per day [38]. The absence of the expected dose response curve (increasing urinary oxalate with increasing ascorbic acid ingestion) can partly be explained by the fact that as the ascorbic acid dose increased, the percentage of urinary ascorbate (presumably an indication of absorption) decreased. An intake of 1 g of ascorbic acid daily resulted in a urinary excretion of ascorbate of 45 %. As the dose increased to 9 g of ascorbic acid daily, the proportion excreted decreased to 18 % [38]. The remaining ascorbic acid is presumably not absorbed since the active absorption mechanism of ascorbate in the gut is saturated. Hughes' study was conducted using normal controls. Obviously, further investigation is necessary using patients with urolithiasis [38].

Possible reasons for the conflicting opinions regarding the potential risk of kidney stone formation after ascorbic acid ingestion are based on the type of data presented [42], the

precautions taken during the study and the methods of analysis, particularly for oxalate [38, 41]. Methodology also includes storage of urine prior to analysis. Ascorbic acid is unstable in the pH range 1 to 12, but within this range it is more unstable at a higher pH [17]. Thus, at higher pH values, the conversion of ascorbate to oxalate occurs more rapidly leading to oxalate values that may be erroneously high. Addition of disodium-ethylenediaminetetraacetic acid (EDTA) as a preservative [17] is considered necessary to lower the pH of the urine and to reduce the conversion of ascorbate to oxalate by chelating the ascorbate in the urine. If a preservative is not used, the data obtained may give erroneously high results. Ascorbic acid encounters both acidic and basic conditions in the gastrointestinal tract and in urine [57], and hence it is of some consequence that a knowledge of the principle metabolic paths of the compound be established as this would be of considerable medical importance. A number of the older techniques used in the measurement of urinary oxalate adjust the pH of the experimental analyte to pH 7 with ethanol and leave the determinations to stand overnight for the precipitation of oxalate as the calcium salt [58]. As discussed, conditions of higher pH result in an increase in the conversion of ascorbic acid to oxalic acid, resulting in erroneous concentrations of urinary oxalate. Certain methods of analysis also require heating of the sample [58]. Experimental work in this area, carried out by Fituri *et al.* [52], indicates that heating the urine sample with raised ascorbate concentrations at 100 °C for 30 minutes results in significant increases in urinary oxalate concentrations, suggesting that the conversion of ascorbic acid to oxalic acid is promoted under these conditions.

Furthermore, the purity of the ingested ascorbic acid preparations used in various studies has been questioned [52, 59]. The presence of tartaric acid in ascorbic acid tablets, may result in higher urinary oxalate since tartaric acid itself is metabolised to oxalate in man [60]. Sucrose is known to increase urinary oxalate [61] and thus increases the risk of urolithiasis. Hence the presence of tartaric acid or sucrose in ascorbic acid preparations may have resulted in exaggerated urinary oxalate levels. It is thus suggested that raised oxalate may be due to assay procedures [52] resulting in *in vitro* conversion of ascorbate to oxalate or perhaps it may be due to the ingestion of impure ascorbic acid.

Investigation into the effects of ascorbic acid have been carried out in both normal subjects and renal stone patients [42]. Chalmers *et al.* [47] observed that recurrent stone

formers had consistently and significantly lower values for ascorbate excretions than did their normal counterparts. These results were unexpected, since from a biochemical point of view, it would have been expected that increased urinary oxalate and ascorbate excretion would be parallel to one another since ascorbate is a major precursor of oxalate [37]. Chalmers *et al.* [47] found that this held for control samples, but that this was not the case in stone forming individuals. These results indicate that there may be an impairment in the handling of ascorbate by stone formers. The increased oxalate and decreased ascorbate in stone formers can be explained in one of two ways :

- there is poor absorption of ascorbate from the gut, and hence unabsorbed ascorbate conversion to oxalate may occur at this site, with subsequent absorption of oxalate, or
- normal absorption of ascorbate by the gut, but there is an increased metabolism of ascorbate to oxalate in the tissues or urinary tract [47].

A subsequent study has shown decreased urinary excretion of ascorbate as well as citrate (both hydrocarboxylic acids) in stone forming individuals [21]. These authors suggest a possible inherent defect in a common carrier which absorbs hydroxycarboxylic acids from the gut. In the study [21], citrate and ascorbate were investigated in conjunction with one another in order to establish whether there is *in vivo* competition between the two for transport by a common carrier in the gut. Results indicated that citrate significantly hindered the uptake of ascorbate into the blood when given a combined load of ascorbate and citrate [21]. Maximal inhibition of ascorbic acid excretion was shown to occur 2 - 3 hours after the ingestion of the combined load. The results suggest a common carrier for the two structurally related anions. In the period 4 - 5 hours after the load, an increase in ascorbic acid uptake may indicate an increased availability of the carrier for ascorbate after the citrate has been cleared from the gut. The inhibition of ascorbic acid absorption by citrate is important in urolithiasis since the presence of citrate in stone forming individuals will enhance the malabsorption of ascorbic acid.

Lewis [cited by 33] has a completely different point of view to most other workers regarding an increase in urinary oxalate levels. He suggests that raised oxalate levels may in fact prevent stone formation in several ways. Firstly, multigram doses of ascorbic acid result in

increased ascorbate in the urine, which will decrease the pH resulting in a decrease in calcium oxalate precipitation. Secondly, ascorbic acid in the urine is free to bind with the calcium ions, thereby reducing the likelihood of calcium oxalate precipitation. Furthermore, the spillage of ascorbic acid into the urine increases diuresis. Since stone formation requires static conditions, the increased flow of urine will decrease the probability of stone formation.

The history of ascorbic acid and oxalic acid is interesting and the possible effects of the two components on urolithiasis is most significant. However, numerous other factors play a role in kidney stone disease. The following section incorporates these interrelating factors of urolithiasis together with a history and background of this disorder.

1.4 Urolithiasis

1.4.1 Historical Background

Renal calculus has been recognised as a medical disorder which can be traced back 6680 years, the age of a bladder stone removed from the mummy of an Egyptian Pharaoh housed in the British Museum [62]. Stone is one of the most common and distressing maladies dating back to pre-Christian times (460 to 370 BC) [63]. Goldman *et al.* [64], give a comprehensive history of stones and the treatment thereof in ancient Egyptian times, the Middle Ages, and in numerous parts of the ancient and more modern day world. Hippocrates, [Joly 1931, cited by 65], recognised both renal and vesical calculus, but would not allow his students to perform surgery. The Hippocratic oath states :

“Neither will I cut them that have stone but will leave this operation to those who are accustomed to perform it”.

The oath resulted in wandering lithotomists who operated on bladder calculi until the end of the 17th century. These aspirant urologists were described by William Clowes (1540 - 1604), surgeon to St. Thomas' Hospital, as “no better than runagates and vagabonds, shameless in countenance, lewd in disposition, brutish in judgement and understanding”. The operators mutilated or killed more than they saved. The operation was only carried out

because the agonies of stone disease were so terrible. Thus, it is not surprising that some stone sufferers resorted to operating on themselves in order to relieve the pain caused by kidney and bladder stone. Possibly the most famous of these is Jan de Doot, a Dutch blacksmith, who removed a 115 g stone from his own bladder whilst his wife was at the fishmarket. He survived the ordeal and today is immortalised in a famous painting which hangs in the Leiden Museum, showing him proudly displaying the stone in his left hand and the knife in his right hand [64].

Surgical methods were developed towards the end of the 17th century, but the operation remained plagued with infection and the difficulty of the surgical removal of stone was prominent, even into the 19th century [64]. The high incidence of urinary calculi and the ghastly nature of lithotomy and its consequences resulted in the search for a means to dissolve stones in the bladder [64]. Herbal remedies and concoctions from plants were recommended from the 1st century for stone disease as well as other ailments, none of which were effective. Patients in England resolved to overcome the distress of stone disease by using the popular and notorious potion devised by Joanna Stevens, who revealed the formula after a sum of five thousand pounds had been paid. It consisted of old tobacco-pipes (clay), eggshells, snail shells, soap, saxifage, burdock seeds and a few other common vegetable extracts [Joly, 1931 cited by 63].

The development of renal surgery for stone was continued through the 18th and 19th century. The application of anaesthesia and antiseptics by the late 19th century resulted in a decrease in mortality as a result of nephrectomy from 80 % to 8 % [66]. In modern-day medicine, numerous treatments to remove kidney stones are performed. Despite the existence of these procedures and the progress in the concepts of calculogenesis, many aspects concerning the cause and prevention of urinary lithiasis remain a mystery.

1.4.2 Epidemiology of Stone Formation

Urinary lithiasis is a world-wide [67], multi-factorial disease [68], the incidence of which has increased dramatically in wealthy industrialised communities during the last century [69]. Renal stone as it occurs in developed countries is twice as prevalent in males than in females,

although the ratio of incidence approaches unity in the earlier and again later decades [70, 71]. The incidence of the disease is influenced by a diverse range of external factors which include geography, climate, environment and nutrition [70].

Table 1.6 shows these and other factors which may influence the formation of urinary calculi [65]. Renal calculi can seldom be attributed to one factor on its own, but rather a combination of these. In spite of these factors, the cause of urinary lithiasis has not been identified, although the factors shown in Table 1.6 have set the scene into which all hypotheses of causation of the condition may eventually fit [70].

Table 1.6 *Factors influencing urinary stone disease.*

| | |
|------------|----------------------|
| Geography | Drinking Water |
| Climate | Drugs and Medication |
| Occupation | Infection |
| Race | Metabolic Disorders |
| Diet | Genetics |

“Stone belts” have been known to exist in particular areas of the same country. In these regions the incidence of stone disease is higher per capita than anywhere else in the country [72]. One of the contributory factors in regions where this occurs, such as the south east of the United States, could be climate. In exceedingly hot areas, dehydration occurs more frequently; if people do not adequately replenish the loss of liquid by increasing their fluid intake [65], stone formation may occur. In hot climates, there is enhanced loss of liquids due to perspiration [73]. This results in a decrease in urinary volume and increase in urinary pH, together with high urine osmolality, higher concentrations of calcium and oxalate and other stone forming urinary constituents [74]. These features contribute to an increased likelihood of stone formation. Exposure to the ultra-violet (UV) portion of the sun’s rays promotes the

formation of vitamin D₃, which increases the intestinal absorption of calcium, calciuria and stone frequency [73, 74]. Climatic effects can however be controlled. A diminished incidence of stone disease was found in populations which had been instructed on the importance of hydration in a hot climate [75]. Indigenous populations of hot desert areas tended to have higher fluid intake and fluid output than immigrants. In geographical considerations of the disease, Anderson (1972) [cited by 70] related the differences in incidence to the development and economy of the countries and communities.

Community surveys in technically developed countries indicate that stone disease is more prevalent in certain occupation groups than in others [70]. Professional groups and sedentary working environments seem to have a higher incidence of stone disease than persons in more active jobs, presumably because the daily routine of the former requires less physical effort [65]. Blacklock showed in 1969 [76] that within the Royal Navy, officers and personnel with predominantly sedentary work showed a higher incidence of stone disease than in the British naval seamen.

Numerous investigators have shown that a non-physiological dietary regimen in the form of insufficient fluid intake [73] and the excessive consumption of foodstuffs containing lithogenic substances may play a crucial role in calculi formation [68]. Anderson has been able to establish that a general relationship exists between economic and cultural development of a country and the type of stone incidence [Anderson, 1967, cited by 63]. A deficiency in animal protein is known to increase the incidence of stone disease. Anderson (1972) [cited by 70] reported a virtual disappearance of this condition in populations with a high incidence of stone disease after the introduction of sufficient animal protein into the diet. From the same source, although not specifically remarked on by Anderson, is that in areas of high sugar consumption per capita, upper urinary tract stone is more common. There are a number of possible links between refined carbohydrate intake and urolithiasis [70]. Hypercalciuria and raised oxalate levels are known to increase the possibility of urolithiasis since calcium oxalate crystals commonly occur in the disease. Lemann *et al.* [77] have shown that an increased intake of glucose or sucrose resulted in an increase in urinary calcium levels in both normal and stone forming subjects, but the calcium excretion levels increased to a much greater extent in the stone forming patients. Increased intestinal absorbance of calcium may also account for an

increased oxalate excretion in the urine since there will be less calcium available for oxalate binding. Increased dietary oxalate may increase excretion of oxalate [5]. As a result patients are advised to avoid foods rich in oxalate [65] (Table 1.3).

The chemical composition of drinking water may also play a vital role in stone formation. "Soft" water is deficient in magnesium, a natural inhibitor of stone formation. It is therefore expected that regions in which water is "soft" may have a higher incidence of urolithiasis. Research has shown that in regions where drinking water is "hard", the incidence of urolithiasis is reduced [78, 79]. Magnesium competes successfully with calcium for free urinary oxalate resulting in the formation of soluble magnesium oxalate rather than insoluble calcium oxalate. Magnesium oxalate is passed in urine with no harmful effects [65]. Some researchers suggest that the chemical composition of water has no effect on urolithiasis [80]. However, there are reports that dietary factors in combination with "water softness" may play a role in stone formation [81].

Certain drugs and medications have side effects which are well known in urinary disorders. Antacid tablets, taken for peptic ulcers, contain silica and many a case has been reported where patients on antacid treatment (magnesium trisilicate oral compound BP) for many years have formed pure silica stones [82, 83, 84].

Urinary infection accounts for 15 - 28 %[†] of all stone types [65]. Infection stones occur more frequently in females [85] since they are more susceptible to urinary tract infection. Westbury [85] has shown that the typical "male" stone is made up of calcium oxalate and the typical "female" stone conforms to the triple phosphate or carbon-apatite pattern, frequently staghorn in appearance.

Certain ethnic groups such as South African blacks, Indians of Mexico and Peru and Eskimos appear to be immune to stone formation. Although diet appears to play an important role in these findings, physiological conditions present in particular race groups cannot be ruled out [65]. Despite the cases which show the genetic predisposition to stone formation in the ethnic groups [86], findings in general detract from the importance of genetic and heredity

[†] An examiner highlighted that a recent series of more than 4500 stones revealed that approximately 10 % were a result of urinary infection (Wilson D.M., J. Urol., 1989, 141:770-774).

influences and indicate more the involvement of environmental factors including dietary customs [70].

1.4.3 Physico-Chemical Factors Governing Stone Formation

According to Finlayson [87], the known physico-chemical features of urolithiasis can essentially be divided into four interrelated processes which include the supersaturation of the urine, nucleation, crystal and particle growth and crystal aggregation.

The thermodynamic driving force for stone formation arises from supersaturation of urine with respect to urinary salts that stones consist of [87]. Ordinarily, urine is supersaturated with respect to calcium oxalate hydrates and calcium phosphate phases [88]. The three hydrates of calcium oxalates, in order of increasing solubility, are monoclinic monohydrate (COM), tetragonal dihydrate (COD) and triclinic trihydrate (COT) [89]. The less stable COT and COD crystals may precipitate and dissolve transiently, acting as precursors for the more thermodynamically stable COM crystals [88]. If stone formation is an extra-cellular process, the level of saturation of the urine will determine whether crystal nucleation, growth and aggregation can actually occur [90]. New crystals do not form in just saturated solutions; in fact a high degree of supersaturation is necessary in order for spontaneous precipitation to occur. Thus a metastable region of supersaturation exists (Figure 1.4.).

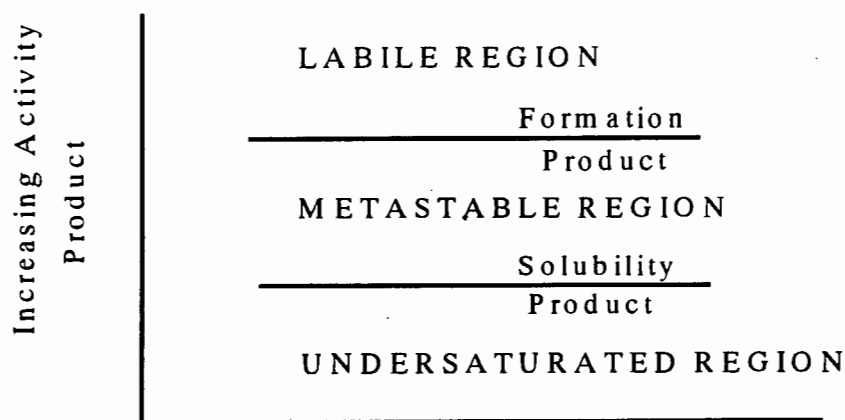


Figure 1.4 *The saturation zones of the urine in relation to the solubility and formation of the salts concerned [90].*

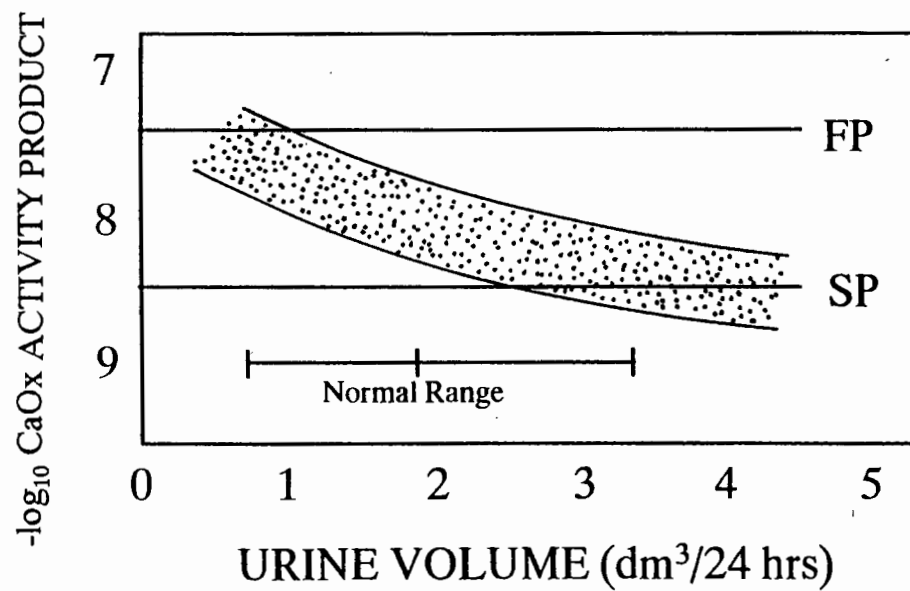
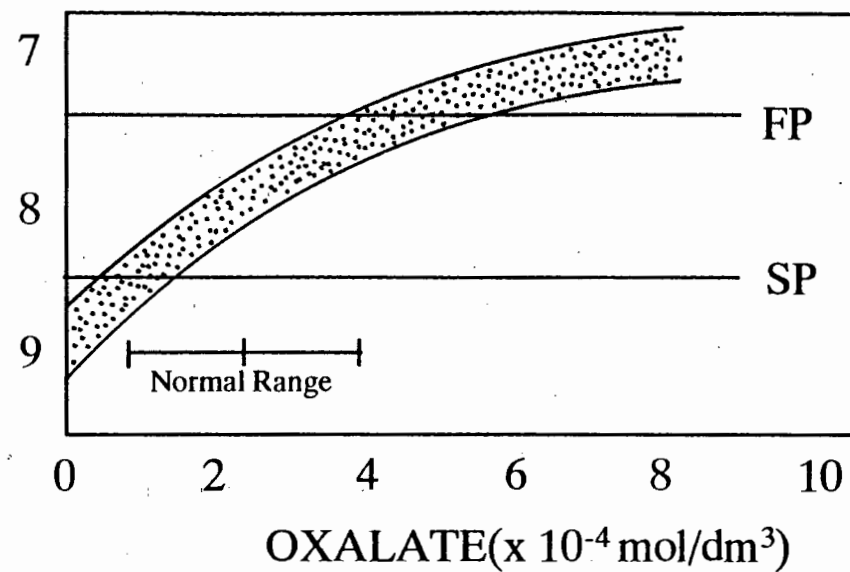
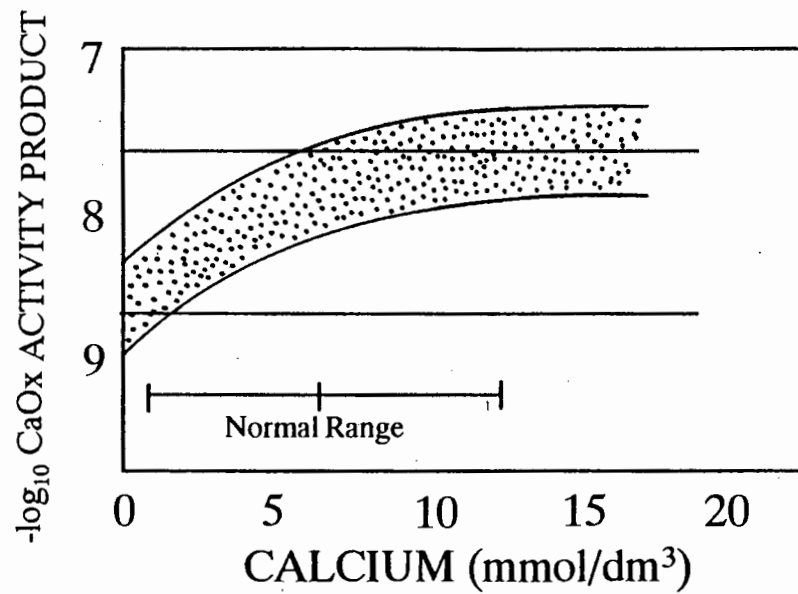
The driving force for stone formation is expressed in terms of free energy and the activities of the unionised salt species (A_i/A_0) in solution at any given condition and at equilibrium, respectively [91]. In order for stones to grow, free energy is required since phase transitions, crystal growth and particle aggregation require free energy [16]. Below the solubility product, the urine is undersaturated and will dissolve crystals of the salt concerned (i.e. the free energy of formation is below zero) [90]. In this case the ratio of the activities of the unionised salt species (A_i/A_0) is less than one [87].

For a given stone salt, saturation of a urine is defined as a driving force equal to one. In such cases, old stones will not dissolve and new stones will not form. However, aggregation of the existing stones can occur, resulting in growth [87].

If the energy of formation is greater than one, the urine is said to be supersaturated. Despite supersaturation, precipitation of stone crystals will not occur if crystals are not already present unless the ratio of the activities of the salts exceed an experimentally determined limit called the "metastable limit" [87]. The metastable zone may thus exist without spontaneous precipitation taking place [90]. If however, some nucleating material is added to a metastable solution, heterogeneous nucleation followed by crystal growth can occur at levels well below the formation product [90]. It is possible for new stones to form spontaneously and old stones to grow above the metastable limit [87].

The three extra-cellular models of stone initiation (Section 1.4.4) require urine to be supersaturated. However, in the case of the "matrix" hypothesis, the level of metastable supersaturation required for crystal growth is considerably lower than in the "inhibitor" and "crystallization" theories, which require a higher levels of supersaturation in order to exceed the level of spontaneous precipitation of the salt concerned. In each cases, once precipitation has occurred, growth and aggregation can take place in the metastable zone [90].

Figure 1.5 *The saturation of urine with calcium oxalate (CaOx) in relation to urinary calcium concentration, urinary oxalate concentration and urine volume [90].*



Calcium oxalate supersaturation is essentially determined by the urinary concentrations of calcium and oxalate [90]. The relative effects of increasing urinary calcium and oxalate concentrations indicate that oxalate is potentially more important than calcium in causing spontaneous crystalluria [90] since more than 50 % of urines in the upper half of the normal range of oxalate concentrations are above the formation product, whereas only small fractions of urines with high calcium concentrations exceed the formation product (Figure 1.5). When the effects of volume are considered, it can be seen that it is very difficult to achieve a urine which is undersaturated with respect to calcium oxalate [90].

Experimental evidence shows that urine of stone formers is supersaturated with respect to the salts from which their stones are formed [22]. However, it has also been shown that the urine of normal subjects is often supersaturated and yet stone formation does not occur. The essential difference must lie in the boundary conditions, the stochastic nature of the process or urinary inhibitors of nucleation, crystal growth and aggregation, or by some as yet undiscovered principle [87]. According to Robertson *et al.* [90], there appear to be two prerequisites for stone formation :

- a critical period of oversaturation and crystalluria during which crystals are formed and retained in the urinary tract and
- a period of metastable supersaturation during which trapped crystals can grow and aggregate into stones.

Nucleation reconciles the increase in free energy when a new surface is formed with the free energy that is lost when a material leaves solution [16]. It is described as the initial event in phase formation and in urolithiasis would correspond specifically with stone salt precipitation [87]. A review of the physical chemistry of nucleation in liquids and solids by Walton [92] develops points of view which have been neglected in the literature of urolithiasis, but according to Finlayson [87] should be pursued in stone research. Nucleation may occur in a number of ways, some of which are listed below [92] :

- classical homogenous nucleation;
- classical heterogeneous nucleation;

- classical coherent and non-coherent nucleation;
- non classical nucleation including
 - * spinodal decomposition
 - * secondary nucleation.

The initial nucleation process, which determines the size, number and morphology of the precipitating crystals, may occur spontaneously [88]. This is homogenous nucleation, and it occurs when the nucleus consists of a pure substance which is presumably crystalline in nature. Heterogenous nucleation occurs when some foreign material trapped in the urinary tract results in the metastable region being narrowed [16]. Heterogenous nucleation on a particle or substrate surface will generally require a lower supersaturation than that required for the induction of homogenous nucleation [88].

Secondary nucleation or the production of additional nuclei in the presence of seed crystals [93] may also be important in the formation of urinary calculi. Secondary nuclei may result from (1) collision breeding, (2) needle breeding, (3) initial breeding, (4) fluid shear which results in particles of existing crystals breaking off due to the movement of solution, or (5) impurity concentration gradients at the surface of the crystals. A comprehensive explanation of these facets are provided by Nancollas [88]. The rate of secondary nucleation is probably governed by the degree of supersaturation since the latter determines the size of the critical nucleus. This allows different proportions of the original nuclei to survive and grow [88].

The size of crystals in the urine of stone formers is significantly greater than the size of crystals in the urine of normal subjects. Additional oxalate in the diet increases the size of the crystals in both groups [90]. Robertson *et al.* [90] established that while normal subjects pass crystals of consistently small size, stone formers' urine contain large octahedral crystals of calcium oxalate dihydrate which are often fused in large aggregates of 40 - 50 μm and occasionally these aggregates grow to 200 - 300 μm . Other studies have shown that the formation and retention of these aggregates in the kidneys plays a vital role in the triggering phase of calcium oxalate stone formation [90]. According to these workers [90], there are two basic differences between the urines of stone formers and those of normal subjects :

- there is a greater degree of calcium oxalate crystalluria in stone formers urine due to higher calcium oxalate supersaturation;
- there exists a difference in the size and type of calcium oxalate crystals between the two groups.

The question now arises as to why there is a difference in crystal size. Chemically, the more saturated a solution, the faster the crystal growth and aggregation rates; hence in the supersaturated urine of stone formers, a faster rate of crystal growth can be expected [90]. However, this is not the only reason for higher crystal growth rate in stone formers. Normal subjects probably excrete some substances in their urine which prevent the growth of large crystals and aggregates. These inhibiting substances are probably deficient in the urine of stone formers [90]. It has been established that stone formers do exhibit less inhibitory activity than normal subjects [19, 88, 94]. Inhibitors of growth and aggregation of crystals in the urine of normal subjects prevent the crystals from reaching a size which may become trapped in the urinary tract. In the absence of these inhibitors, crystals form spontaneously once the formation product of the salts has been exceeded. Crystal growth and aggregation then occur, resulting in the formation of particles which may indeed be large enough to become trapped in the urinary tract. The particle will continue to grow even if the urine saturation falls to within saturation levels of the metastable limit. The factors which will influence the growth of a stone lie in the balance of the precipitability of the stone components and the inhibitory activity of the urine [90].

Growth history of stones can be speculated from studies of particle growth in urine. Conventional crystal growth experiments involve metastable supersaturated solutions of calcium oxalate or phosphate being dosed with seed crystals and the rate of the reaction determined by measuring the decrease in concentration of lattice ions as a function of time [88]. Since the different calcium oxalate phases may form over a wide range of supersaturations resulting in formation and dissolution of crystal phases during the reaction, it is impossible to quantify the kinetics of mineralisation [88]. In particle growth studies it is necessary to distinguish between crystal growth and crystal aggregation [87].

Flocculation refers to the tendency for small suspended particles in a liquid to form aggregates or agglomerates [88]. Aggregates are considered to be groups of primary particles joined at their faces and having considerably less surface area than the constituent particles. Aggregation may be a very rapid process depending on the size and morphology of the crystallites [88]. Agglomeration is regarded as the joining of particles at the edges and corners, resulting in the surface area of the resulting particles being similar to the constituent particles.

Aggregation is important in urolithiasis [87] since it was observed that normal subjects frequently passed single crystals whereas stone formers passed large crystal aggregates. However, relatively little work has been carried out on this aspect of urinary crystallization [88]. Finlayson [87] outlines aggregation as it pertains to urolithiasis in the following way. Particles of large diameter (1 cm or more) are more susceptible to the force of gravity than to forces of adhesion. However, as the size of the particles diminishes, adhesion becomes more prevalent than gravitation. The susceptibility of adhesional forces in particles of diameter 1 μm is approximately one million times higher than the gravitational force on such particles [Rumpf *et al.* 1977, cited by 87]. Thus when dealing with fine particles it is essential that forces of adhesion be taken into account. In crystallization studies it becomes necessary to consider both particle-particle and particle-membrane adhesion [Finlayson, 1976, cited by 87]. The stability of minute crystalluria in urines is determined by the nature of the forces which are effective only at short distances of separation. When the surfaces are brought close together, London forces or Van Der Waals forces predominate, resulting in overall attraction and natural flocculation. Surface charges do play an important role and suspensions are rendered stable by repulsive electrostatic forces [88].

1.4.4 Mechanism of Stone Formation

As stated earlier, calcium oxalate stone formation is related to crystallization processes in the urine [95] which can be subdivided into four stages [96]. Firstly, there must be a period of high supersaturation [87, 96] which results in crystal nucleation (increase in crystal number).

This is followed by a period of rapid crystal growth and aggregation in which the primary particles increase in size [95, 96].

Numerous hypotheses have been put forward in an attempt to explain the formation of stones in the renal tract. Robertson *et al.* [90] have suggested that any hypothesis should account for the formation and retention of some critical nucleus and its subsequent growth. Stone growth arises as a result of an imbalance between saturation of the urine with stone forming substances and the activity of crystal inhibitors [94]. The hypotheses which have emerged may be subdivided into two groups : firstly, those which describe the formation and retention of the stone nucleus as an intracellular process [97] and secondly, those which define stone formation as taking place entirely within the lumen of the urinary tract (i.e. an extracellular phenomenon) [90]. The latter group comprises three possible theories which can be summed up as follows :

- matrix hypothesis;
- crystallization inhibitor hypothesis;
- excessive supersaturation of the urine leading to spontaneous precipitation hypothesis.

The matrix hypothesis invokes the active role of non-crystalline, organic material which provides structural rigidity and hence provides a framework for mineralisation [98, 99]. Stone development is initiated by some chemical or pathological process which promotes the precipitation of stone forming salts by secretion of specific crystalline substances into the urine.

The crystallization inhibitor theory suggests that stone formation and growth may occur due to a deficiency or absence of specific ions which may inhibit the crystallization of stone forming salts. The urine of normal subjects contains sufficient of these inhibitors whereas stone formers urine is deficient in these protective substances [100, 101].

Finally, there is the theory that the urine stream is excessively saturated with one of the stone forming salts which may lead to spontaneous precipitation of the salt independent of a pre-formed matrix or inhibitors of crystallization. Persistent crystalluria may result in the trapping of an aggregate or large crystal in the urinary tract, resulting in stone formation [22,

102]. This is known as the free particle theory [96]. Alternatively, the rate of production of crystals in the narrow tubules may cause a blockage due to crystal overload [90], resulting in a primary particle becoming attached to the walls of the renal system through the participation of some gluing substance [96]. This is the fixed particle theory [96]. Subsequent growth of the trapped particles occurs due to aggregation of the passing materials. Together with the adsorption of protein on the growing crystal faces, this process may produce a fully-developed calculus [90]. In both theories a nidus forms which constitutes the centre around which a stone will form by the continued processes of crystal growth and adhesion [96].

1.4.5 Urinary Risk Factors of Calcium Nephrolithiasis

Calcium renal stone formation occurs as a combined result of a number of individual determinate factors in urine [7]. In recent years the term *risk factor* has been associated with the phenomenon. It has emerged that a number of the urinary risk factors contribute to the increased likelihood of precipitation of calcium oxalate and calcium phosphate stones. The urinary risk factors as summed up by Ryall *et al.* [7] can be considered as those determining the degree of calcium oxalate saturation in the urine and those which, once the crystal nucleation has been initiated, actually allow for crystals to enlarge and aggregate [7].

According to Robertson *et al.* [73], the 6 main risk factors are high pH and low urinary volume, raised oxalate and calcium renal output, the absence of substances which retard crystal growth (inhibitors) and an increase in uric acid excretion. These risk factors, together with other pertinent factors regarding stone growth, are listed in Table 1.7.

The solubility's of calcium oxalate and calcium phosphate are related to the pH [7]. However, there is controversy in the literature as to the significance of urinary pH on stone formation. Although studies which compare the urinary pH in normal controls and stone formers are few [7], most agree that there is no noticeable difference in the pH of each group [22, 23, 103, 104]. In contrast, Robertson *et al.* [19] show that there is a difference in urinary pH between stone formers and normal controls and hence conclude that urinary pH is a risk factor in calcium oxalate urolithiasis. Although there is undoubtedly an effect of urinary pH on the solubility of calcium stone salts, the conflicting opinions in the literature indicate that

caution must be carefully exercised when classifying urinary pH as a risk factor in stone disease [7].

Table 1.7 *Risk factors for calcium stone formation [73].*

| |
|----------------------|
| urinary pH |
| volume (dehydration) |
| hyperoxaluria |
| hypercalciuria |
| hyperuricosuria |
| inhibitors |

An increase in urinary volume results in a lower concentration of stone constituents, thereby reducing the probability of stone formation. However, the increase in volume also results in the dilution of urinary inhibitors [7]. It is thus not a simple matter to ascertain the effects of volume on urolithiasis. There is evidence which indicates that stone formation is significantly lower in a population whose average urinary output was a third higher than in a second control population [75]. Volume is thus influential in calcium urolithiasis, but stone formers do not necessarily exhibit a lower urinary output than do normal individuals [22, 23, 104]. This is not to say that urinary volume is not a risk factor in calculi formation, “theory and common sense indicate that it is” [7], but that there isn’t anything to be gained by volume measurement between stone formers and control subjects. Urinary volume was not one of the original list of risk factors [19] but was included later [18]. Possibly the most pragmatic approach to urinary volume is to let common sense prevail : the higher the fluid intake, the greater the dilution of the urine and hence the lower the chance of stone formation [7].

Urinary inhibitors are naturally occurring components of the urine which have been shown in model crystallization systems to prevent or retard the nucleation, growth or

aggregation of calcium oxalate crystals [7]. Stone formation may occur as a result of the absence of natural inhibitors, which in normal subjects, prevent the formation of crystalluria [88]. The studies as summarised by Robertson *et al.* [94] have centred around the effect of inhibitors of new crystals in the urine, rather than on the growth and aggregation of pre-existing ones. According to Ryall *et al.* [104], if inhibitors do affect crystal aggregation, they may exert an indirect influence on the growth of crystals by reducing the amount of surface area available for the deposition of new material. Robertson *et al.* [96] also state that the urine of controls as well as stone formers can attain high levels of supersaturation in which the inhibitors have little or no effect on the spontaneous formation of new crystals. Thus inhibitors may not prevent the nucleation of crystals [Fleisch *et al.* 1965 cited by 94, 105] but rather prevent the growth and aggregation of existing crystals. Since growth and aggregation occur in the urines of stone formers, it is suggested that these urines are deficient in such substances [94].

In order to determine the importance of urinary components in preventing mineralisation, studies have been made in conventional crystallization experiments. A number of low molecular weight urinary components such as magnesium, citrate and pyrophosphate as well as higher molecular weight compounds such as glycosaminoglycans (GAGS) have been shown to play roles as effective inhibitors of nucleation and crystallization [7, 106, 107].

Pyrophosphate was the first urinary inhibitor to be isolated from urine and identified [100]. The effect which pyrophosphate was observed to have on preventing calcification indicated its possible inhibitory role [7]. A dichotomy of opinion exists in the literature as to the potential of pyrophosphate as an inhibitor in human urine [101, 108, 109, 110]. Once again, the conflict in the literature indicates that although pyrophosphate may not be a major determinant in urolithiasis, it does play a minor role in calcium oxalate urolithiasis [7].

Citrate has been observed to directly inhibit calcium oxalate crystal growth [111] and aggregation [112, 113, 114]. Citrate may influence crystallization properties in a number of ways. Complex formation between citrate and calcium ions decrease the ion-activity products of both calcium oxalate and calcium phosphate. The resulting change in supersaturation brings about a reduction potential of crystal formation and growth [114]. However, the literature

depicts conflicting opinions in this regard, with reports indicating that there is no apparent difference in citrate excretion of stone formers and normal control subjects [Pak *et al.* 1978, cited by 7, 22]. On the other hand, other investigations have shown that there is an abnormally low urinary citrate excretion in the stone forming individuals [115, 116, 117] relative to normal controls. Even in situations where a significant reduction in citrate concentrations in stone formers was not shown, a decided decrease in the ratio of citrate/calcium excretion was observed in the stone forming individuals [118]. Administration with alkaline citrate has proven to be effective in correcting low citrate concentrations in stone forming individuals [119, 120]. It can therefore be suggested that the urinary excretion of citrate may be influential in determining the likelihood of calcium stone formation.

Magnesium is a known inhibitor of urolithiasis afflictions. The ability of magnesium to increase the solubility of calcium oxalate was recognised in 1929 by Hammerstan. More recent studies on rats [Rushton *et al.* 1982, cited by 7] and in human urine [121] have shown magnesium to reduce calcium oxalate crystal formation. It has several possible modes of action, the first of which is that high urinary magnesium concentrations form complexes with oxalate. As a result, the ion activity product of calcium oxalate is reduced resulting in a lower risk of calcium oxalate precipitation [5]. These effects are attributable to competition by the magnesium with calcium for the oxalate ion, resulting in the formation of the more soluble magnesium oxalate [7]. If however, magnesium is a critical factor in the formation of urinary calculi, it might be expected that abnormally low levels of magnesium would occur in the urine of stone forming individuals. This has not been unequivocally demonstrated in the literature. Investigations which indicate that magnesium excretion is indistinguishable between stone formers and their normal counterparts [22, 122] are more common.

Presently, evidence indicates that macromolecules are the principle contributors of inhibitory activity [123]. The bulk of inhibitory activity of calcium oxalate crystal growth has been attributed to GAGS [112, 124] and proteins [Worcester, 1987, cited by 7]. As a result, investigations have been carried out in order to establish whether stone formers excrete lower concentrations of either of these inhibitory substances. Robertson *et al.* [19] showed that GAG output is lower in the urine of stone formers than in the urine of control subjects which was later confirmed in investigations by Baggio *et al.* [125, 126]. Other studies have indicated

that there is no difference in the GAG output between stone formers and control subjects [104, 127]. Very little data is available on the effects of proteins as urinary inhibitors. Whether proteins do or do not play a role in calcium oxalate disease is therefore open to debate [7].

Other workers have suggested that the presence of macromolecules in urine [128] indicates that they may in fact play a promotory role in stone formation [9]. Indeed this is supported by some *in vitro* studies. However, current evidence is insufficient to state with certainty whether macromolecules in human urine play a promotory or inhibitory role [7].

It has been found that monosodium urate forms a nidus for calcium oxalate stone formation, or growth by means of epitaxy [71]. As a result, uric acid is considered to be a risk factor in calcium oxalate stone formation.

A history of stone disease in one or more first degree relatives has been known to increase the tendency for stone formation by 25 % in patients with recurrent stone disease [71]. Dehydration causes urine to become more concentrated and is thus an obvious risk factor [71].

The nature of urine is complex and hence factors that may have an effect on urine will be intricate. All the aforementioned risk factors may result in an increase in the risk of the stone forming potential of urine. However, the importance of each factor in urolithiasis is clouded by uncertainty and differing opinions. As a result, further investigation is required into the intricate network of possible urinary risk factors in order to ascertain the true effects of each individual component as a risk in calcium oxalate nephrolithiasis.

1.5 Objectives of Research

The existing investigations of megadose ingestion of ascorbic acid present a conflicting picture as to whether ascorbate promotes hyperoxaluria or not and, in so doing, whether it increases the risk of calcium oxalate urolithiasis. The possible relationship between ascorbic acid ingestion and kidney stone formation is somewhat alarming in today's health conscious society where self prescription megadose ingestion of this substance is commonplace in the management of a vast range of diseases as diverse as the common cold and cancer [39]. Although the concentration and excretion ratios of oxalate and ascorbate have been measured in many different studies involving ascorbic acid ingestion, workers have failed to consider a host of urinary risk factors in assessing the role of dietary ascorbic acid but have confined their investigations to the effects on urinary oxalate excretion only. The present study is aimed at investigating whether there is a significant urinary stone risk associated with megadose ingestion of ascorbic acid on a daily basis by focusing attention on many risk factors.

Many of the reported studies failed to take into account the possibility of *in vitro* conversion of ascorbic acid to oxalate, leading to a lack of confidence in reported oxalate values. Moreover, investigators have confined themselves to employing only one method of ascorbic acid analysis and have not checked it against others.

The objectives of the study are as follows :

- to determine the influence of dietary ascorbic acid on a host of calcium oxalate stone formation risk factors;
- to determine urinary oxalate (and other components) in the presence and absence of an EDTA preservative so as to establish realistic values for these components and to assess the necessity of a preservative;
- to design, develop and test a flow-injection system for analysis of urinary ascorbic acid;
- to determine urinary ascorbic acid by the aforementioned flow-injection method as well as by a manual titration method.

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Chapter 2.

General Theory of Techniques

Many techniques can be applied in the study of crystalline material and hence in the study of urinary calculi. This chapter deals briefly with some of the basic principles of the techniques used in the present investigation, including atomic absorption, enzymatic assays of oxalate and citrate, turbidimetry and Coulter multisizer. Various techniques for the determination of ascorbic acid in biological samples are also discussed.

2.1 Analytical Visible Spectrophotometry

Spectrophotometric methods for the determination of elements are based on the absorption of visible radiation. Essentially, spectrophotometric methods depend on the relationship between the absorption of radiation by a solution and the concentration of the coloured species in solution. In the application of spectrophotometric methods, the species analysed is often converted to a coloured complex. In cases where the analytical species is colourless, indirect spectrophotometric methods can be used for determination. Alternatively, analysis by ultraviolet (UV) spectrophotometry can sometimes be used [1].

Spectrophotometric techniques are remarkably versatile, sensitive and precise. Application is broad, covering most elements with the exception of the noble gases. A wide range of concentrations can be determined, from macroquantities to traces [1]. Spectrophotometry is one of the most precise instrumental means of analysis and is probably one of the most widely used single procedures in chemical and clinical laboratories world-wide [2]. Furthermore, the basic apparatus required, a spectrophotometer, is relatively inexpensive.

Attempts to use the colour of a complex in analytical analysis dates back to ancient times. Some investigators believe the origin of colorimetry to date back to 1852 with the formulation of Beer's fundamental laws of colorimetry (spectrophotometry). Some colorimetric methods dating back to the 19th century, such as the determination of bromide in

natural waters by oxidation and extraction of the resulting bromine with ether, are still in use today. Originally colorimetric methods were determined in colorimetric test-tubes. Towards the end of the 19th century, visual colorimeters with filters were applied and towards the 1930's, the first photoelectric colorimeters were employed. Since then, photoelectric apparatus has been developed and improved [1].

Originally colorimetric techniques involved the comparison of analyte concentration to a range of standards in order to determine the analyte concentration. Today, photoelectric instruments rely neither on measurement nor comparison of colour, but rather on the fraction of incident radiation that is absorbed by the coloured complex. The term spectrophotometry is thus more popular than colorimetry [1].

2.1.1 Absorption Laws

When a parallel beam of radiation of intensity I_0 passes through a layer of coloured solution, the intensity of the emergent radiation (I) will always be less than the intensity of the incident radiation (I_0) since of some of the energy (I_a) is absorbed by the particles, some energy is transmitted (I_t) and some is reflected (I_r) and scattered by the walls of the cuvette [1] (Equation 2.1). Since measurements are usually made with respect to reference solutions in the same cuvette, the last term can be neglected since it is constant.

$$I_0 = I_a + I_t + I_r \qquad \text{Equation 2.1}$$

Absorption of the incident beam is dependant on the thickness of the coloured layer (i.e. radiation pathlength (b), and the concentration (c), of the absorbing species [2] (Figure 2.1).

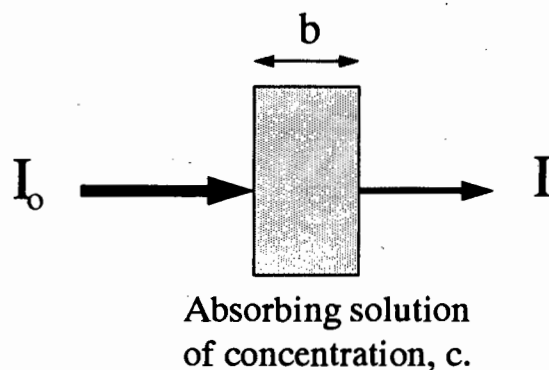


Figure 2.1 *Diagram showing the incident beam passing through an absorbing solution [2].*

Bouguer established the relationship between pathlength, b , and concentration, c , of the absorbing species in 1729. Lambert formulated this mathematical relationship in a more accurate manner in 1760. Beer determined the linear relationship between the absorption of the radiation and the concentration of the light absorbing component of the solution in 1852 [1]. Despite the mathematical input from Bouguer and Lambert, the relationship is commonly called Beer's Law.

$$T = I / I_0 \quad \text{Equation 2.2}$$

$$\text{and, } A = -\log_{10} T = \log (I_0 / I) = \epsilon b c \quad \text{Equation 2.3}$$

where T = transmittance

A = absorbance

I_0 = intensity of incidence radiation

I = intensity of transmitted radiation

c = concentration

ϵ = absorptivity

b = pathlength

Thus if the concentration, c , of the absorbing species is doubled and the pathlength is reduced by a factor of 2, and assuming the total number of absorbing species remains constant, the absorbance will remain constant.

If a solution contains more than one absorbing species, the total absorbance (A_t) is equal to the sum of the component absorbances (Equation 2.4). Provided the pathlength remains constant and there is no interaction between absorbing species, the law of additivity holds [2].

$$A_t = \epsilon_1 bc_1 + \epsilon_2 bc_2 + \dots + \epsilon_n bc_n = b (\epsilon_1 c_1 + \epsilon_2 c_2 + \dots + \epsilon_n c_n)$$

Equation 2.4

Deviations from Beer's Law do occur. It is important that the system is tested to ensure that Beer's Law holds. A coloured species obeys the Law if a straight line passing through the origin is obtained for a plot of absorbance versus concentration. The linear range in which the absorbing species obeys Beer's Law can then be determined visually from the plot.

2.1.2 Deviations from Beer's Law

In theory, the relationship between concentration and absorbance is linear (Figure 2.2). In practise, however, positive and negative deviations from linearity do occur which may be physical, chemical or instrumental in nature [2].

Chemical deviations from Beer's Law may occur due to chemical changes associated with concentration of the absorbing species or due to interaction of the absorbing species with the solvent solution. Beer's Law only applies in dilute solutions, which places restrictions on analysis. This a result of the electrostatic nature of concentrated ions which affects the absorptivity and causes departures from Beer's Law. Some deviations are fundamental (e.g. stepwise formation of complexes). Homogenous solutions are essential since turbid solutions will give rise to light scattering [1].

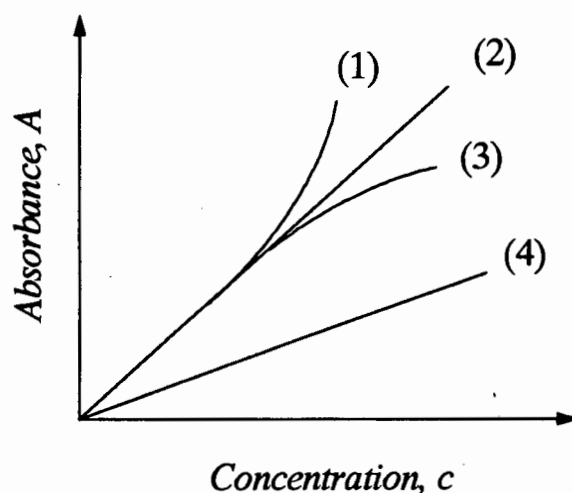


Figure 2.2 Graph showing the linear relationship of Beer's Law (2) and the positive (1) and negative (3) deviations from the Law and deviation independent of concentration (4).

Physical and instrumental factors result in departure from Beer's Law, since the law only applies in conditions of monochromatic light. Polychromatic light sources, in conjunction with filters and gratings, are commonly employed in routine chemical analysis to ensure monochromaticity. In these conditions, deviation from Beer's Law is not appreciable [1, 2].

Poor quality absorptometers result in deviation from Beer's Law due to failure of a light sensitive element to respond in a linear fashion to incident radiation. Stray light due to scattering from imperfections in instrumental optical components such as prisms, lenses and filters also result in departure from Beer's Law. In cases where scattered light is of the order of the incident radiation, there is a marked decrease of absorbance, however this is only likely to occur at high absorbances [1, 2]. Despite potential areas in which it could occur, deviation from Beer's Law is rare. Correction factors may be used to accommodate deviations, however this is undesirable.

2.1.3 Photoelectric Apparatus

The fundamental apparatus used for measuring the absorbance of coloured solutions includes spectrophotometers and photoelectric colorimeters with filters. The basis of operation for both types is provided by the photoelectric effect [1]. A block diagram of a photoelectric effect-based detector is presented in Figure 2.3.

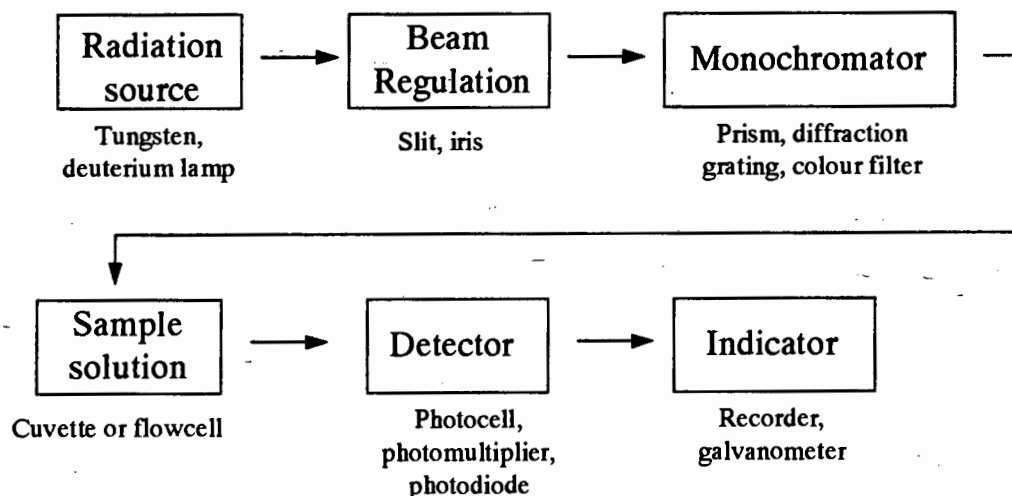


Figure 2.3 Block diagram of a photoelectric effect-based detector [1].

The simplest, most common spectrophotometer is a single beam spectrophotometer in which the reference and sample solutions are analysed in the same light path successively. Radiation sources ideally give a continuous spectrum over the widest possible wavelength range. Spectrophotometers are fitted with a monochromator capable of isolating a radiation band that for practical purposes is monochromatic (0.1 - 2 nm band width). A monochromator is fitted with an element to disperse light e.g. a prism or diffraction grating and two narrow slits. A source of white light is focused on the entrance slit of a monochromator so that a narrow band of light at the specific wavelength is selected. Light is passed through the second slit. The single wavelength light then passes through the sample in the cuvette (flowcell in FIA) to the detecting device. The absorbance of the sample is subsequently obtained and recorded [1].

2.1.4 Spectrophotometric Techniques

There are a wide variety of spectrophotometric techniques available for application to any number of chemical systems [1]. Three such techniques have been applied in this investigation :

- **Standard curve technique** : By applying Beer's Law, the linear relationship between the absorbance and concentration of a species is expressed graphically to be the standard curve for the chemical species. Sample absorbance is then translated into concentration by means of the standard curve.
- **Flow-injection analysis** : Flow-injection is a technique based on injection of a sample into a continuously flowing reagent stream. Application of flow-injection analysis with spectrophotometry yields a fast, precise and versatile means of analysis and detection. Flow-injection analysis is capable of handling microlitre analyte volumes (10 - 200 μ l).
- **Turbidimetry** : Spectrophotometric methods involve the measurement of the light absorption (or scatter) by suspensions of sparingly soluble solids.

2.1.5 Quantitative Visible Spectrophotometry

UV-visible absorption spectrophotometry is one of the most useful techniques of analysis available for quantitative analysis [2]. The important characteristics of spectrophotometric and photometric methods are outlined below :

- **Wide applicability** : An extensive range of chemical compounds and elements, both organic and inorganic, absorb UV and visible radiation, and hence are amenable to direct quantitative determination. Many non-absorbing species can be manipulated into an absorbing derivative and this technique employed.
- **High sensitivity** : Typical detection limits range from 10^{-2} to 10^{-5} %.

- Moderate to high selectivity : Often a wavelength can be identified at which only the analyte absorbs, thus avoiding preliminary separations. Use of additional wavelengths in cases where overlapping absorption bands occur can overcome the need to separate analytes.
- High accuracy* : Relative errors associated with this method of detection are usually in the range 1 - 5 %. These errors can be reduced further by applying special precautions.
- Ease and convenience : Spectrophotometric and photometric measurements are easily and rapidly performed with modern instruments. In addition, the methods lend themselves to automation.

2.2 Turbidity

Turbidity is the expression of an optical property which causes the light to be scattered and absorbed rather than transmitted in straight lines through the sample [3]. It is difficult to correlate the turbidity of a solution with the weight concentration of the suspended matter due to limiting factors such as particle size, shape and the refractive index, all of which affect the light scattering properties of the suspension [3].

2.2.1 History of Turbidity

The measurement of turbidity was initiated by Whipple and Jackson in 1900 [4]. The investigation led to the development of a series of standard suspensions prepared from diatomaceous earth and the scale derived in parts per million (ppm) is still used in the calibration of turbidimeters today. One of the earliest turbidimeters was the Diaphanometer which consisted of a graduated glass tube with a cross shaped window at the bottom. The

* Accuracy is the closeness of the measured analyte concentration to the true concentration of the analyte. Precision is the reproducibility of the results obtained in numerous determinations of the same analyte concentration.

sample was slowly poured into the glass tube while observing the image of the cross from the top. As the cross disappeared into a uniform glow, the sample was no longer poured into the glass tube and the turbidity reading was taken from the graduated scale of the glass tube. The candle turbidimeter developed by Jackson and still employed today, used a candle as a light source in place of the cross window, but the extinction principle is the same [4]. Low range turbidimeters for continuous measurement were not available until after 1958. The development of low range turbidimeters resulted in a stimulus for rapid rate filtration and is very useful in the water industry as a means for determining the dosage rate of clarification chemicals in water, resulting in less chemical waste [3].

2.2.2 Jackson's Candle Turbidimeter

Turbidity measurements by the candle turbidimeter are based on the light path through a suspension that just causes the image of the flame to become indistinguishable against the general background illumination when the flame is viewed through the suspension. The longer the light path, the lower the turbidity.

The candle turbidimeter consists of a glass tube calibrated according the light path (cm) and Jackson Turbidity Units (JTU) (Appendix B), a standard candle and a support which aligns the tube and the candle in a vertical manner. The candle is supported by a spring loaded system which is designed to ensure that as the candle burns away, it is pushed up to the top of the support. The top of the support for the candle is 7.6 cm from the bottom of the glass tube. The bottom of the glass tube is flat, of highly polished optical glass and conforms to specifications for Nessler tubes. It is graduated to read directly in JTU. The glass tube is enclosed in metal during readings to prevent breakage, but more importantly to exclude extraneous light. To ensure uniform results, the flame should be kept at a constant size and distance from the bottom of the glass tube. This is achieved by frequent trimming of the wick and ensuring that the candle is continuously pushed to the top of its support. Conditions should be draft free to ensure that there is no flickering of the flame. The candle should also not be burnt for more than a few minutes at a time since this leads to an increase in flame size [3].

2.2.3 Nephelometric Turbidimeters

The nephelometric method is based on comparison of the intensity of light scattered by a sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions. The higher the intensity of scattered light, the higher is the turbidity. Formazin polymers are generally employed as normal reference turbidity standards. They are easy to prepare and give reproducible light scattering properties [3].

Nephelometric instruments consist of a turbidimeter which has a light source for illuminating the sample and one or more photoelectric detectors with a readout device to indicate the intensity of the scattered light at 90° to the path of incident light. It is important to note that differences in design from one instrument to the next result in variances in measured values for turbidity even though the same suspension is used for the calibration [3].

Commercial turbidimeters for the determination of low turbidities generally give a good indication of light scattering in one particular direction, usually at right angles to the incident beam. These turbidimeters (or nephelometers) are relatively unaffected by small changes in design parameters and hence are specified as standard instruments for measuring low turbidities. Non-standard nephelometers are more sensitive to larger particles and are useful for process monitoring [3].

Standard Methods (13th Edition) [4] gives preference to nephelometric photoelectrical instrumental methods as standard methods of turbidity determination, although the Jackson's candle visual method remains as an alternative standard of turbidity measurement [4].

There are two basic types of photoelectric turbidimeters : (1) nephelometers and (2) absorptometers. The differences between the two turbidimeters are detailed in Table 2.1.

Table 2.1 *The differences between nephelometers and absorptometers [4].*

| Nephelometers | Absorptometers |
|---|--|
| Can be highly sensitive for measuring small turbidities | Not sensitive to small turbidities |
| Zero signal at zero turbidity | Maximum signal at zero turbidity |
| Direct response - signal increases with increasing turbidity | Negative response - signal decreases with increasing turbidity |
| Linear response at low turbidities and possible linear response at higher turbidities | Disobeys Beers Law giving a non - linear response, even in moderate ranges |
| Dissolved colour does not register as turbidity although some colours may give a negative error | Dissolved colour registers as turbidity |

2.2.4 Limitations of Turbidimeters

The standard method to determine turbidity is based on Jackson's candle turbidimeter. The Jackson's candle can handle a wide range of samples and, as yet, no turbidimeter is able to replicate this for all samples [3]. However, limitations do exist. A candle flame is used and light in the yellow-red range wavelength is radiated. Very fine particles do not scatter or reflect light in this range with the result that fine particle suspensions cannot be measured using a Jackson's candle turbidimeter [4]. The lowest turbidity value which can be measured is 25 units [4], which is often too high for experimental use in the study of solution suspensions such as water and calcium oxalate crystal nucleation and growth. Fine suspensions can be measured by means of a photoelectric turbidimeter in which an incandescent light source is employed. Furthermore, Jackson's turbidimeter cannot measure black particles since the absorption of light is so great compared to the scattering of light that the field of view becomes dark before sufficient sample is poured into the tube to reach an image extinction point.

Interferences occur in nephelometric instruments and Jackson's candle turbidimeters due to dirty glassware, air bubbles and the effect of vibrations that disturb surface visibility of the sample solution [3].

2.2.5 Units of Turbidity

There have been numerous changes over the years regarding the unit of measurement and the expression of turbidity. Initially turbidity units were expressed as parts per million (ppm) turbidity (1946). However, by 1955 the term "ppm" was discarded since turbidity was described as an expression of the "optical property of a sample which causes light rays to be scattered and absorbed rather than transmitted as straight lines through the sample" [4]. The unit term for turbidity thus simply became turbidity units (TU). In the water industry, the units are referred to as Jackson's turbidity units (JTU) [4]. In the case where formazin is used in standardisation of the turbidimeter, the units are referred to as formazin turbidity units (FTU) [4] and in nephelometric measurements the units are called nephelometric turbidity units (NTU) [3].

2.2.6 Standards and Standard Development

Standard suspensions for turbidity measurements were initially prepared using materials in nature, such as diatomaceous earth used by Whipple and Jackson in 1900, and later supplemented by Fuller's earth, kaolin and sediment from stream beds. Standards were prepared by addition of the material to distilled water. The turbidity was then determined by Jackson's turbidimeter and further standards were then prepared from the stock solution by appropriate dilution. Formazin, developed in 1926, was found to be an almost ideal turbidity material and is now adopted by the water industry and Standard Methods (13th Edition) as the standard turbidity material [4].

2.2.7 Shortcomings of Turbidimetry

The most serious disadvantage of photoelectric turbidity measurements concerns stray light. Any scratches or imperfections in the glass window, any dirt, film or condensation on the glass will cause light to scatter. Some of this light reaches the photocell, with the result that positive deviation to turbidity measurements is observed [4]. This is a serious problem, particularly when measuring low turbidity values. The problem of dirty glass windows has been overcome by pouring molten glass directly onto a molten metal surface when the window is manufactured. The effect is superior polished glass surfaces on both sides of the window, which does not normally occur in routine glass manufacture. If the light source enters through the upper surface of a water sample in the nephelometer, the problem of scattered light is overcome. The adoption of this principle resulted in turbidimeters which could read very low and very high turbidities [4].

There is a misunderstanding concerning the natural desire to relate the concentration of suspended matter with the measured turbidity of the sample. It is only possible to compare samples of the same material, measured on the same instrument. Even then, a linear relationship is not always observed between the concentration of suspended matter and FTU turbidity measurements. An example of this is the turbidity of a natural sample which measures 500 FTU, but after 1 + 4 dilution with distilled water, may read more than the expected 100 FTU. A possible explanation is that on dilution of some types of solutions, particles may sub-divide into a larger number of smaller particles resulting in higher turbidity readings [4].

There is very little correlation between turbidity measurements obtained with different types of turbidimeters and Jackson's instrument [4]. Indirect secondary methods are sometimes required to estimate turbidity. Optical systems have fundamental differences and hence employ different types of secondary instruments. Turbidities will thus vary from one instrument to another despite their being pre-calibrated against the candle turbidimeter [3]. Further discrepancies occur in turbidity analysis due to the use of different suspensions for the preparation of instrumental calibration curves. A standard reference suspension which has reproducible light scattering properties is needed for turbidimeter calibration. Even then, when two or more turbidimeters are standardised using the same turbidity standard (e.g. formazin),

they will usually give very different readings for the turbidity of a sample containing some other turbidity substance. Variations under these circumstances can be as much as 500 % [4].

2.3 The Coulter Multisizer

The Coulter multisizer is a flexible, multichannel, particle size analyser developed by Coulter in 1956 [5]. It is designed to give high resolution and precision over a wide range of sample sizes and materials [5]. The basic principle of operation allows the determination of the number and size of particles suspended in a conductive liquid. This is achieved by monitoring the electronic current between two electrodes immersed in the conductive liquid on either side of a small aperture, through which the suspension of the particles is forced to flow [6]. As each particle is drawn into the aperture, it displaces its own volume of electrolyte solution and its presence is detected by modulation of the current path [7] (i.e. the changes in resistance as particles pass through the orifice generate voltages, the amplitudes of which are proportional to the volumes of the particles [8]). The series of pulses is electronically scaled, counted and accumulated in a series of size related channels which, when displayed, produce a size distribution curve. The instrument is designed to provide accurate particle size distribution curves over a wide range of either diameter or volume.

The technique was originally applied to blood cell counting and later modified to allow counting of bacterial cells [8]. Kubitschek [cited by 8] showed that Coulter methods could be used in the measurement of cell-volume distributions as well as number counting. Modified instruments were soon developed with which particles could be sized as well as counted [8]. Robertson [9] initiated the use of an electronic particle counter for counting and sizing calcium crystals in urine. Calcium crystalluria may be expressed as either the number or the volume of crystals excreted per unit volume of urine when using this technique. In addition, number-size and volume-size distributions of the crystals may be obtained [9]. The method was found to be reproducible and capable of counting all calcium crystals present in a urine sample. Coulter multisizer techniques have been applied extensively in research related to crystalluria [10, 11, 12, 13, 14, 15]. This technique provides a means of studying both particle growth and particle

aggregation mechanisms which cannot be distinguished by other techniques.

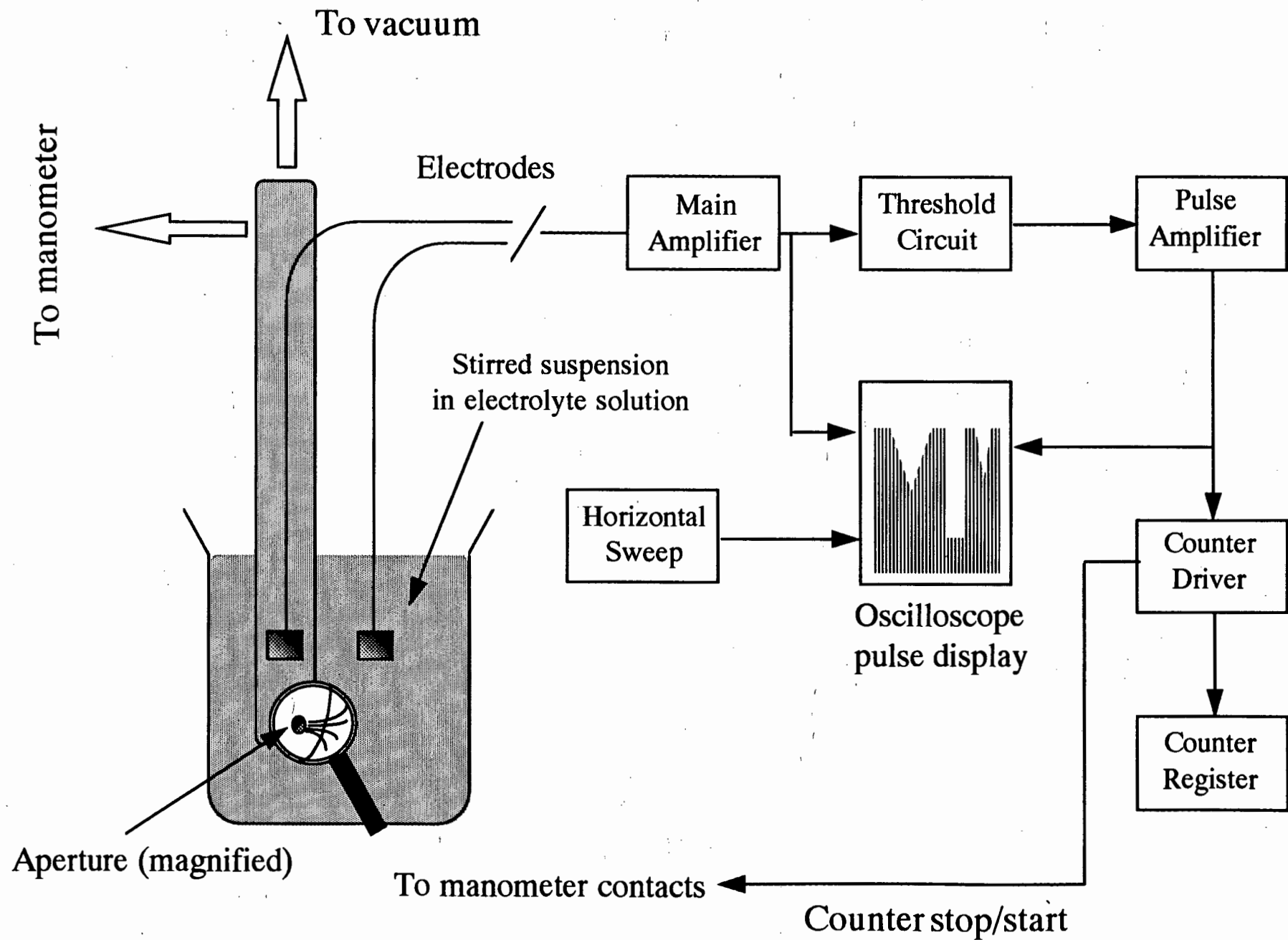
2.3.1 Basic Principles of Operation

The operating principle of the Coulter multisizer can be followed by referring to Figure 2:4. A controlled vacuum initiates flow through an orifice in a glass tube which unbalances a mercury siphon. The system is then isolated from the vacuum source by closing a tap. Flow continues due to the balancing action of the mercury siphon. The advancing column of mercury activates the counter by means of start and stop probes, placed such that a known volume of electrolyte passes through the orifice (500 μl in the present study). Resistance across the orifice is monitored by means of immersed electrodes on either side. As each particle passes through the orifice it changes resistance, generating a voltage pulse which is amplified, sized and counted. The size distribution of the suspended phase is determined [8] from the derived data.

The amplified voltage pulses are fed to a threshold circuit which has an adjustable threshold level. All pulses above the threshold level are counted and this count represents the number of particles larger than a determinable volume proportional to the appropriate threshold setting. Some instruments have upper and lower threshold circuits which permit sizing between two determinable volumes (i.e. a relative-frequency distribution). By taking a series of set amplifications and threshold settings, data are obtained directly for determining number frequency against volume [8].

The instrument offers a wide range of operating modes which can be selected to obtain optimum results from the samples to be analysed. In order to minimise operator involvement, the machine is equipped with several automated calibration procedures for standard orifice tube sizes. Furthermore, the more usual modes of operation are set into the instrument's memory and are recalled when the "automatic" mode of operation is selected as opposed to the more flexible "manual" mode. Calibration constants and automatic operations can be set in the memory, and this new data can then be accessed in the "automatic" mode [8].

Figure 2.4 *Schematic diagram of the well known electrical sensing zone, or Coulter principle, illustrating the basic operating principle. Adapted from Lloyd [7].*



2.4 Calcium Oxalate Crystal Sedimentation in Urine

In principle, kidney stone formation can be due to either crystal growth or aggregation, or by the combined growth and aggregation of small crystals into large particles of clinically significant size [16]. It is difficult to distinguish between crystal growth and aggregation and hence techniques to determine each individually are difficult to effect.

Hess *et al.* [17] developed a method to measure aggregation of calcium oxalate monohydrate (COM) crystals in the absence of COM crystal growth, based on the terminal sedimentation velocity of COM crystal aggregates produced by slow stirring of an equilibrated COM crystal suspension [Blomen, 1982 PhD thesis, cited by 17]. COM crystal slurries were prepared in which crystals and their bathing solutions were allowed to come to equilibrium, so that crystals no longer grew or dissolved. The outer diameter[†] (OD) of the COM crystals was determined at a wavelength of 620 nm (OD_{620}). A 2 cm³ aliquot of the solution was transferred to a 1 cm light path quartz cuvette in a cell holder thermostated at 37 °C by a constant temperature waterbath. In order to show the stability of the slurries, absorbance was measured for a period of 10 seconds to obtain a stable baseline value. Crystal aggregation was induced by slow magnetic stirring using a teflon-coated magnetic stirrer bar at a stirring rate of 500 rpm [17].

During the stirring at 500 rpm, the number of particles forced down by gravitational forces on the system equal the number of particles forced upwards by the stirrer [Blomen, 1982, PhD thesis cited by 17]. However, the OD_{620} initially decreased in a hyperbolic fashion, only stabilising after 180 s (Figure 3.5). The decrease in OD_{620} in the first 180 s can only be due to crystal aggregation since the equilibrium solution does not allow for crystal growth or dissolution. At 180 s, stirring was stopped and spontaneous sedimentation of particles in the suspension was monitored for 300 s. After the stirring was stopped, the OD_{620} decreased.

[†] Outer diameter (OD) is the term of measurement employed by Hess *et al.* [17]. OD is equivalent to absorbance, the units of which are absorbance units (AU). However, the theory discussed in this study has conformed with the format of the original reference.

This decrease is interpreted as being due to particle settling, which is controlled by particle size.

The theoretical approach for interpreting the data was presented by Hess *et al.* [17] as follows. Assume a system of evenly distributed, free falling spheres of identical size; the constant terminal velocity (v_t) will be equal to the rate of sedimentation.

$$v_t = l / t \quad \text{Equation 2.5}$$

where l is the sedimentation distance in the fluid column and t is the time required for all the particles to reach the bottom.

In the system developed by Hess *et al.* [17], OD_{620} decreased progressively and fell linearly at its maximum rate, between 50 and 150 s after the start of the sedimentation (Figure 3.5). The linear portion of the curve has a turbidity slope (t_s) equal to OD'/t_o where OD' is OD_{620} at the beginning of the linear portion of the curve and t_o is the time in which OD_{620} would fall from OD' to 0 if particles settled at a maximal constant rate. In other words, t_o is obtained as the x-intercept of the graphical extrapolation of the linear portion of the curve to $OD_{620} = 0$, minus the time at the start of the linear portion, t' . Thus if t (Equation 2.5) is substituted by OD'/t_s then,

$$v_t = (l t_s) / OD' \quad \text{Equation 2.6}$$

In ideal spheres the terminal velocity is equal to $(2/9)(1/\eta)(\delta_1 - \delta_2)r^2g$ at low sedimentation rates where η is the viscosity of the solution, δ_1 and δ_2 are the densities of the particles and fluid respectively, r is the particle radius and g is gravitational acceleration. Thus,

$$v_t = (l t_s) / OD' = (2 / 9) (1 / \eta) (\delta_1 - \delta_2) r^2 g \quad \text{Equation 2.7}$$

Since t_s is a function of r^2 in a given solution, the greater the value of t_s , the larger the COM crystal aggregates. Terminal velocity is thus determined from the turbidity slope of a plot of OD_{620} vs time (Equation 2.6) together with the particle sedimentation distance derived from the cuvette used. Particle radius can be determined from Equation 2.7, while the density and viscosity values were determined by Hess *et al.* [17] from the supernatant of a COM crystal slurry. The published density value for COM crystals [18] was used in the calculations of the work carried out in [17].

2.5 Methods for Ascorbic Acid Analysis

The analysis of ascorbic acid presents numerous difficulties. A routine method of analysis which is free from interferences does not exist [19]. Techniques for ascorbic acid analysis should ideally allow for the vitamins many oxidative products to be distinguished so that accurate determination of the compound can be achieved. At the very least they should allow for simultaneous determination of ascorbic acid and its oxidative products with a minimum of sample preparation and interference from any other chemical species which may be present. The situation is further complicated since ascorbic acid generally co-exists in plant and animal tissues with numerous other organic compounds from which it has to be separated. Separation procedures can be bypassed if a unique property of the ascorbic acid can be measured. L-ascorbic acid exhibits redox behaviour, a property which is used extensively in the analytical determination of the compound [19].

2.5.1 Titrimetric and Colorimetric Methods of Analysis.

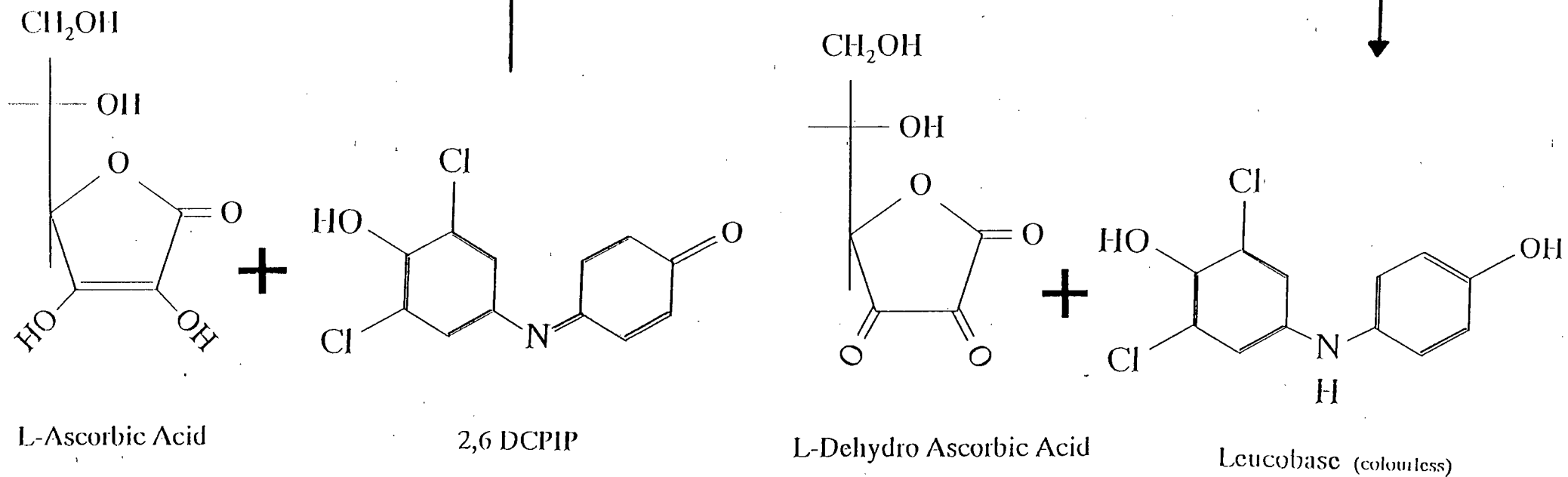
Titrimetric analyses of L-ascorbic acid are based on the reducing properties of the 1,2-enediol group [20]. Since 1927 [19], the technique which has been extensively employed is oxidation using 2,6 dichlorophenolindophenol (2,6 DCPIP) [20], a dye which is blue in neutral conditions, pink in acidic conditions and on reaction with ascorbic acid forms a colourless solution [19].

The procedure developed by Tillman *et al.* [cited by 21] and outlined by Harris *et al.* [21] involves adjustment of a test solution to near neutrality using sodium acetate followed by titration with the indicator until reduction is complete. The specificity of the method has been found to diminish in the presence of other reducing substances and unsatisfactory results are obtained if the analysis is carried out in neutral solution [21]. A method using the same indicator was eventually modified by Harris *et al.* [21] which, when tested against naturally occurring reducing substances such as glutathione and pyrogallol, showed consistently negative results. The solution to be analysed was first acidified to pH 2.5 by the addition of concentrated acetic acid. The indicator was then added to a suitable volume of the analyte until it was no longer reduced. The technique is more precise if a specific volume of the indicator is placed into a titration vessel and a volume of the unknown titrated against the fixed volume of the indicator, as is carried out by Gowenlock *et al.* [22].

Redox titration of ascorbic acid with 2,6 DCPIP in an acid solution allows for the reduction of the dye to a colourless leucobase while the ascorbate is oxidised to dehydroascorbate [22] (Figure 2.5). If dehydroascorbate is already present in the solution, it is necessary to reduce it to ascorbic acid in acid solution by the addition of hydrogen sulphide. Any other reducing substances which may be present in urine, may reduce the specificity of this technique. This method is widely used in titrimetric analysis to determine urinary ascorbic acid concentration. It is applied in university chemistry laboratories and in research laboratories. The method is employed at a number of the South African Hospitals, including Groote Schuur Hospital and Red Cross Childrens Hospital, Cape Town.

Figure 2.5 *Chemical formulae for the reaction of 2,6 DCPIP with L-ascorbic acid [23].*

REDUCTION



OXIDATION

Numerous other methods, which employ the strong reducing properties of ascorbic acid, exist to determine ascorbic acid concentration in substances. Other techniques require time consuming laboratory preparations of the samples and the analysis itself is time consuming. As a result, the method usually used to determine ascorbic acid is that originally developed by Tillman *et al.* [cited by 21] previously described. The method is relatively simple and can be employed in conditions in which there is a high concentration of ascorbic acid [19]. In most cases, the end point is easily detected. However, there are disadvantages to the technique. It is susceptible to the interference of other reducing agents such as cysteine and glutathione which are frequently present in biological fluids, iron (II) which is commonly present in food stuffs and sulphites which are added as preservatives to soft drinks [20]. As a result, the technique lacks specificity. There are treatments for some of the reducing substances in individual cases but there is no single application which can remove all reducing substances in an analyte solution. A further disadvantage of the technique occurs in coloured solutions, such as urine, in which the endpoint can be masked. As a result, the endpoint is more difficult to ascertain, resulting in instrumental means of detection such as polarography being utilised [19].

Another titrimetric method employs chloramine-T ($\text{MeC}_6\text{H}_4\text{SO}_2\text{NNaCl}$) as a stable titrant for ascorbic acid determination [20]. The reagent is used with either acidified potassium-iodide and starch or in the presence of acidified potassium-bromide and methyl red. Iron(II) does not interfere in either method. However, interference due to sulphites and sulphydryls occurs and can be overcome in both cases by pre-reaction with acrylonitrile. Both reactions allow for the determination of ascorbic acid (without dehydroascorbic acid) in the presence of iron, sulphites and sulphydryls without interference after the pre-treatment of samples with acrylonitrile.

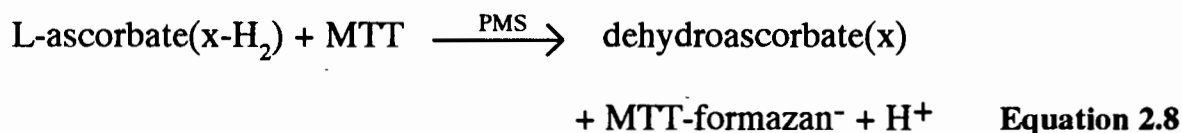
Other indicators have been employed in titrimetric analysis of ascorbic acid. These include the use of ferrozine α, α' dipyridine, 2,4,6-tripyridyl-s-triazine [19] or 2,4-dinitrophenylhydrazine [22, 24]. Another titrimetric determination of ascorbic acid involves the use of cerium(IV) in a phosphoric acid medium using a ferroin sulphate indicator [25]. The kinetics and mechanism of the oxidation reaction of ascorbic acid with cerium(IV) in sulphuric acid solution has also been investigated [26].

Dehydroascorbic acid is often assumed to be the only product of the redox analysis. However, further products of oxidation do exist in the solution. A method to determine the concentration of L-ascorbic acid as well as dehydroascorbic acid and all further oxidation products in a manner which is unambiguous, accurate, sensitive and precise still eludes investigators [19].

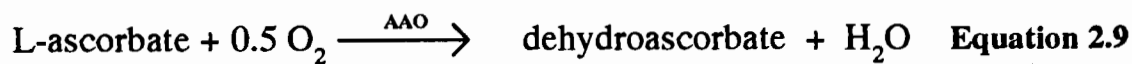
2.5.2 Enzymatic Colorimetric Analysis

Boehringer Mannheim have developed an enzymatic kit (Cat. No. 409 677) for the analysis of ascorbic acid in foodstuffs and other materials [27] based on the reaction developed and described by Beutler [28].

L-ascorbic acid and some reducing substances ($x-H_2$) reduce the tetrazolium salt, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), in the presence of the electron carrier, 5-methylphenazinium methyl sulphate (PMS), at a pH of 3.5, to a formazan. Equation 2.8 shows the reaction taking place in the cuvette in which the sum of the reducing substances is measured [27].



A sample blank is necessary for the analysis of ascorbic acid concentrations when using this technique. In order to prepare a sample blank, it is necessary to remove any L-ascorbate which may be present in the sample. Oxidative removal of L-ascorbate is achieved using ascorbate oxidase (AAO) [27] (Equation 2.9). Other oxidative products, such as dehydroascorbic acid, do not react with MTT/PMS and it is unnecessary to remove them in the development of a suitable sample blank [27].



The absorbance difference of the sample minus the absorbance difference of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The MTT-formazan is the measuring parameter and is determined by means of its absorbance in the visible range at 578 nm [27].

2.5.3 Spectrophotometric Analysis

Aqueous ascorbic acid is colourless and hence there is no significant absorption in the visible spectrum. However, neutral solutions of ascorbic acid do have a strong absorbance at 265 nm which allows for direct spectrophotometric analysis. Ascorbic acid is usually present in solutions which contain other substances which absorb in this range, and subsequently this technique is limited [19].

The molar absorptivity of ascorbic acid at 265 nm varies considerably because it undergoes rapid atmospheric oxidation. Thus, it is essential that anaerobic conditions are maintained, otherwise there may be significant differences in the absorptions at this wavelength. These difficulties are exacerbated by the presence of several transition metal ions, such as copper(II), which catalyse the oxidation. EDTA can be used as a chelating agent in solutions which contain transition metal ions.

In an attempt to overcome the problem of interferences from biological solutions which contain ascorbic acid, specific colour reactions have been used to determine L-ascorbic acid and/or its oxidation products. The titrimetric analysis of ascorbic acid using 2,6 DCPIP dye is one reaction which has been adapted for colorimetric determination. Other compounds and indicators have been used for colorimetric analysis, but the specifics have not been detailed in this study.

2.5.4 Electrochemical Methods

Electrochemical techniques offer selectivity, precision and accuracy as well as simple operative mechanisms. Methods have been devised [29], but further development of work is required. These methods of analysis have not been widely adopted.

2.5.5 Chromatographic Techniques

It is not yet possible to determine small amounts of ascorbic acid in a sample solution, or infact to distinguish between its other oxidative products. Chromatography is used to separate analytes from one another in solution and thus allows for this specificity [19].

Gas-liquid chromatography is the most common chromatographic method in general use. However, it is not suitable for ascorbic acid analysis, since the vitamin is not volatile. Sample work-up in order to convert ascorbic acid to its more volatile tri-ether is laborious. The method does deliver accurate, reproducible results, but is time consuming.

High performance liquid chromatography (HPLC) is a more convenient method of analysis and has been applied in the determination of catecholamines, of which ascorbic acid is one [29]. Despite its successful determinations of organic and inorganic compounds as well as some vitamins, it is not ideal for ascorbic acid analysis. Numerous problems remain and it cannot be used in all circumstances with equal ease, for a variety of matrices and for very low concentrations. Detection of ascorbic acid in HPLC depends very much on a physical property specific to ascorbic acid and its oxidative products. Absorption in the UV-visible range has been used, but is much less sensitive to dehydroascorbic acid due to its low molar absorptivity.

2.5.6 Flow-Injection Analysis

Numerous conventional methods, which are described above, have been used in the determination of ascorbic acid, each of which has its own advantages and limitations [30].

Flow-injection analysis (FIA) has been applied twice in the determination of ascorbic acid using electro-analytical detection [30]. Strohl *et al.* [31] used a reticulated glassy carbon electrode to carry out the coulometric and amperometric determination of ascorbic acid. The coulometric determination allowed for the ascorbic acid to be completely electrolysed, enabling it to be determined without a calibration graph. The sampling frequency decreased with respect to the amperometric method. Bradberry *et al.* [32] were successful with an amperometric method using enzymatic reactors to determine the vitamin in the tissues of the brain.

A photometric flow-injection technique was developed by Lazaro *et al.* [30] using reagents detailed by Verma *et al.* [20] in the manual titration of this method. This technique was then specifically applied to the determination of ascorbic acid in urine [33]. The principles and chemistry of the starch-potassium iodide flow-injection system developed by Lazaro *et al.* [33] were applied to the system in the following manner. The ascorbic acid sample, dissolved in an acidic medium, is injected into a chloramine-T carrier stream after merging with a starch-iodide stream. Iodide forms HI in an acidic medium, which subsequently reacts with chloramine-T resulting in the formation of iodine (Equation 2.10).



The iodine formed oxidises the ascorbic acid. Once the ascorbic acid has been fully oxidised, the iodine binds with the starch which constitutes the indicator reaction. Sulphuric acid can be considered the blank in the reaction since sample preparation is carried out in a sulphuric acid medium to prevent the precipitation of the reagent when the sample is injected. The absorbance is at a maximum for sulphuric acid in the absence of ascorbic acid. The presence of ascorbic acid reduces the analytical signal proportional to its concentration [33].

Very recent documentations on flow-injection analysis methods include the assay of ascorbic acid using cerium(IV) [34]. An ascorbic acid solution was injected into a carrier stream of cerium(IV) prepared in sulphuric acid. A mixing chamber was employed for the

resultant solution which was then propelled through a flow cell and detected by means of a spectrophotometer at a wavelength of 410 nm. Recently Sultan *et al.* [35] have developed a new method of analysis for ascorbic acid. A simple colorimetric flow-injection method was developed for the assay of ascorbic acid in drug formulations [35]. Ascorbic acid was injected into a flowing stream of iron(III) and then mixed with 1,10-phenanthroline in sulphuric acid media. The mixture was allowed to react in a reaction coil and the resulting colour of the 1,10-phenanthroline-iron(II) complex was monitored at 510 nm.

2.5.7 Closing Comments

Ideally an accurate and reliable method to determine the concentration of ascorbic acid excreted in urine is required. Documentation of a reliable and efficient means to carry out this determination is limited. As mentioned earlier, investigation into the methods used in some of the South African hospitals indicate the most popular method remains the manual titration of a urine sample with 2,6 DCPIP. The method is employed in other institutions internationally (University College Hospitals, London). The method however is laborious and time consuming and in conditions where ascorbic acid is present in the urine in low concentrations, the end point of the titration is exceedingly difficult to ascertain by human eye. Automation of this method using an analytical means of detection (e.g. visible spectrophotometry), would therefore be advantageous.

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Chapter 3.

Materials and Methods

This chapter explains the experimental procedure and apparatus used in the study. The ascorbic acid protocol and the urine collection procedure are discussed. Details of the urinary component analysis, crystallization experiments and risk ratio indices are given. Finally, the method of statistical evaluation of the results obtained in the study is described.

3.1 Participants and Protocols

A survey of the literature regarding ascorbic acid doses that have been administered in previous studies led to the selection of a protocol followed by Hatch *et al.* [1]. In private correspondence with these workers, it was ascertained that the most severe side effect experienced by participants in the original study was laxative action, which is common in megadose ingestion of ascorbic acid.

“Scorbex” (Lennon Ltd., South Africa) ascorbic acid tablets were used in the present study. The manufacturers confirmed that this product contained only pure ascorbic acid and binding agents and that tartaric acid and sucrose were not present.

3.1.1 Ascorbic Acid Protocol

The protocol initially decided upon involved the ingestion of 8 g ascorbic acid per day for 9 consecutive days. However, the first participant in this protocol developed haematuria after 8 days. A full description of this particular case is presented in Chapter 5.

Following the detection of haematuria in the first participant of the investigation, the ascorbic acid protocol was revised. Each participant was required to ingest 4 g of ascorbic acid daily for a period of five days.

Participants were all post-graduate students in the Department of Chemistry at the University of Cape Town (U.C.T), in the age group 20 to 30 years. None had a history of stone formation. Participants were required to ingest 1 g ascorbic acid four times daily : at breakfast, lunch, early evening and approximately one hour before retiring. They were encouraged to maintain a high fluid intake and to pass urine regularly throughout the protocol.

Each participant provided two consecutive 24 hour specimens immediately prior to ascorbic acid ingestion which served as control samples (samples 1 and 2). After commencing the ascorbic acid protocol, participants collected 24 hour urine samples during the first, third and fifth day of the ingestion period (samples 3, 4 and 5 respectively). After five days, ingestion of ascorbic acid was stopped but participants continued to collect 24 hour urine samples on alternate days during the following five days (samples 6, 7 and 8 respectively).

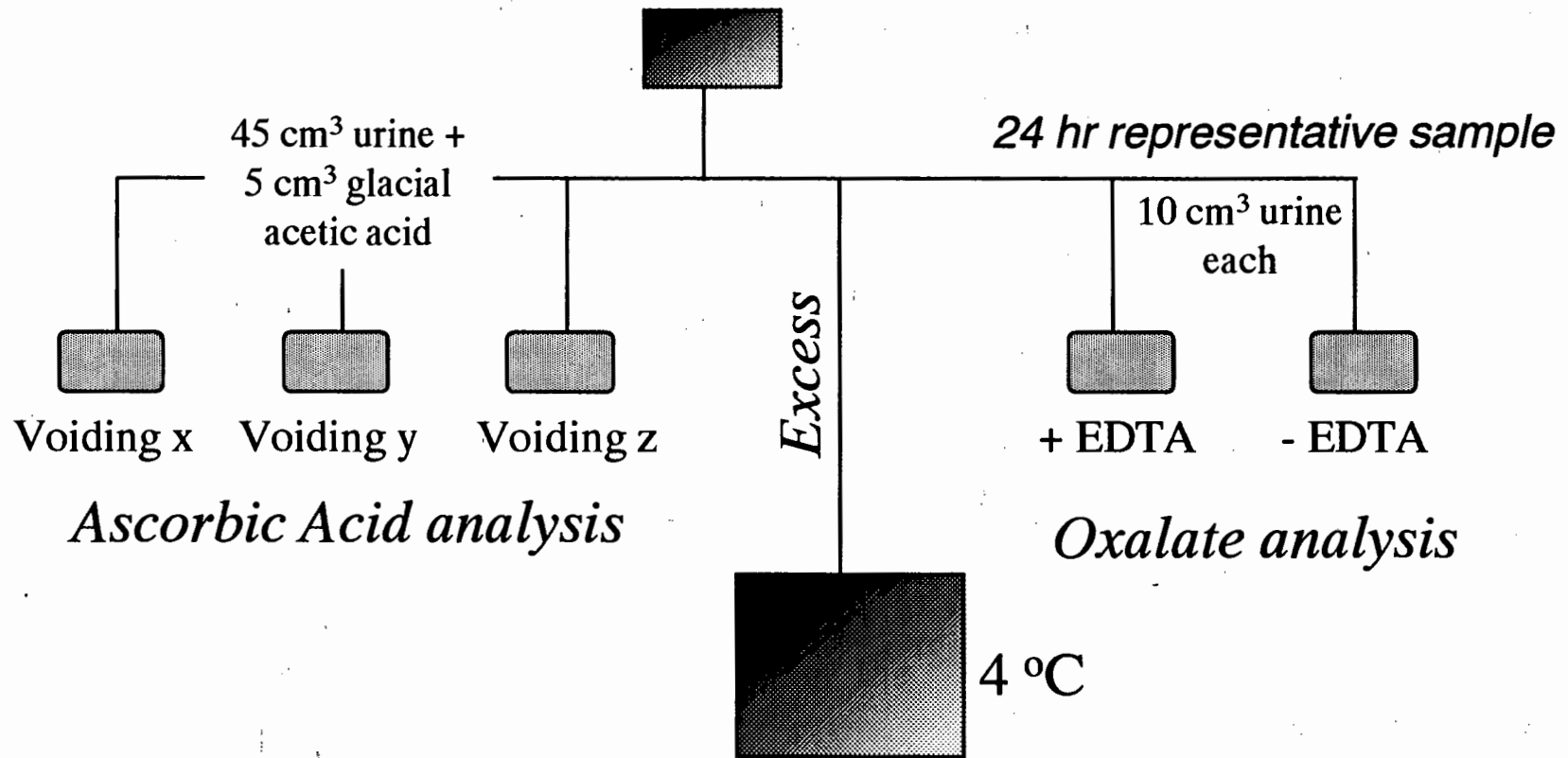
3.2 Urine Collection Procedure

All urine samples were collected over a period of 24 hours. Participants were required to collect each voiding in a clean glass beaker and to pipette 10 cm³ thereof into a small glass bottle containing 0.38 g EDTA. The final concentration of EDTA was 10 mmol.dm⁻³ [2]. The EDTA acts as a preservative to minimise the *in vitro* conversion of ascorbic acid to oxalate during the collection period [2, 3]. Participants was also required to pipette a further 10 cm³ of urine into a second glass bottle, but no preservative was present. These samples will henceforth be referred to as the "24 hour representative samples with (+) or without (-) EDTA".

In addition, participants were required to add 45 cm³ of the freshly voided urine into a clean glass bottle containing 5 cm³ glacial acetic acid for immediate ascorbic acid analysis [4]. These samples were analysed within a few hours of voiding. Three such aliquots were effected during the 24 hour collection period : at the second, "x", and fourth, "y", voidings of the day and at the first, "z", voiding of the following morning (Figure 3.1).

Figure 3.1 *Urine collection procedure followed by each participant.*

Collection



*Urine composition, Oxalate analysis,
and crystallization experiments.*

After performing the aforementioned procedures, excess urine was poured into a clean 2.5 dm³ glass bottle which contained no preservative. This specimen is henceforth referred to as the "bulk 24 hour sample".

The bottles were cleaned by soaking in soapy solution overnight and then thoroughly rinsed with distilled water before being soaked in ~ 5 % nitric acid for a 24 hour period. Final rinsing was achieved with distilled water. The smaller glass bottles, beakers and pipettes were washed in hot water and soaked overnight in ~ 10 % nitric acid. The glassware was then thoroughly rinsed with distilled water and allowed to dry before being used for further urine collections.

3.3 Urine Analysis

Urines were analysed on a daily basis as follows. The samples were checked for infection using Boehringer Mannheim dipsticks (Combur-10-Test). The 24 hour representative sample with EDTA was diluted to 100 cm³ with isotonic saline [2] to bring the concentration of EDTA to 10 mmol.dm⁻³. The volume and pH of the bulk urine sample and the two representative samples were measured and recorded.

Aliquots of each sample (representative samples and bulk 24 hour collection) were analysed for chloride, phosphate, urate and creatinine by private pathologists. In those cases where urine samples contained concentrations of ascorbic acid greater than 1136 µmol.dm⁻³, urate could not be determined due to interference [5, 6]. Other methods employed for urate analysis such as phosphotungstic acid (PTA) are also subject to interferences by ascorbic acid [7].

Aliquots of 30 cm³ of the bulk sample and the two representative samples were filtered through a 0.74 µm sieve to remove all cellular debris and proteinaceous material. A small quantity of each was subjected to atomic absorption analysis (VARIAN Techtron Flame Atomic Absorption Spectrophotometer 5, 1970) for calcium [8], potassium, magnesium [8] and sodium. The remaining volume of each aliquot was used for the determination of oxalate

(Sigma Kit, Procedure No. 591) and citrate (Boehringer Mannheim UV method, Cat. No. 139 076) by enzymatic assay.

3.4 Metastable Limit Determination

Nucleation of calcium oxalate (CaOx) crystals can be induced by dosing the urine with aqueous sodium oxalate (NaOx). The extent of crystallization can be monitored by several different methods, including measurement of the turbidity of the urine [9, 10] or by determining crystal number-size distributions with a Coulter multisizer [11, 12, 13, 14, 15, 16]. Initially turbidity or crystal numbers increase slowly in response to increasing NaOx concentration but when the metastable limit (MSL) is reached, these values increase dramatically. Readings of turbidity (or crystal number) are plotted as a function of the concentration of the administered NaOx dose concentration. The minimum amount of oxalate necessary to induce nucleation (detected by the sudden increase in particle number or turbidity) is taken to be the measured limit of metastability of the urine, determined by interpolation of the line to the abscissa [16] (Figure 3.2).

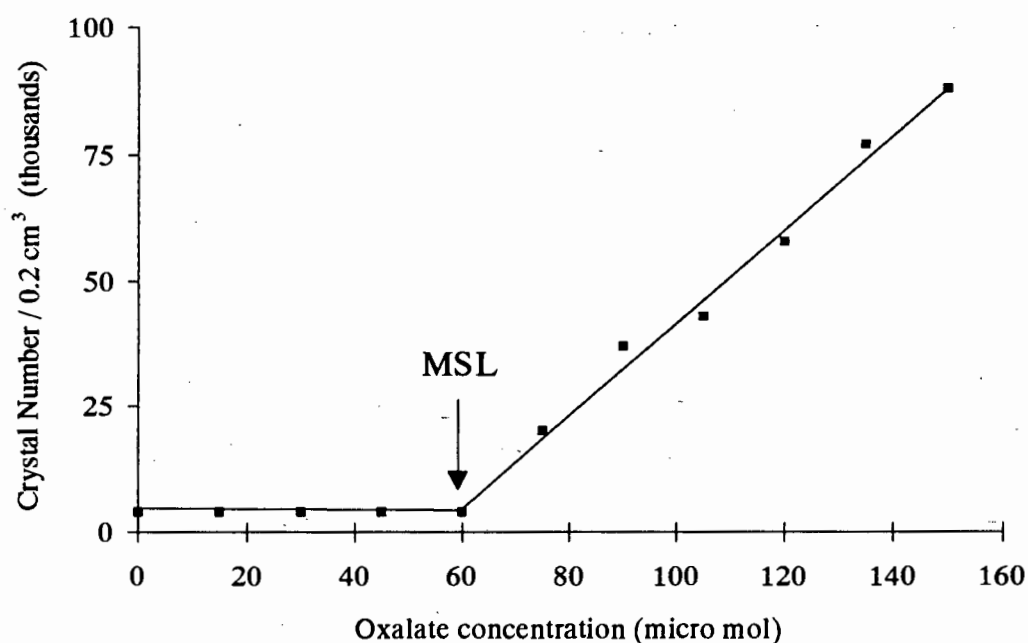


Figure 3.2 *Determination of the practical metastable limit of urine [16].*

3.4.1 *Metastable Limit by Turbidiometry*

A volume of 800 cm³ of the bulk urine was filtered through Sartorius glassfibre prefilters followed by filtration through Sartorius cellulose acetate filters (0.45 µm) to remove all crystalline and non-crystalline matter. Aliquots of 30 cm³ of this filtered urine were pipetted into each of 12 narrow necked plastic bottles which were then incubated for a period of 30 minutes in a MEMMERT oven at a temperature of 37 °C.

Aliquots were dosed pairwise at five minute intervals with 0.3 cm³ of aqueous sodium oxalate (NaOx) solution of increasing concentration (range 0.01 - 0.2 mol.dm⁻³ (*M*); Appendix C) and incubated for a further 30 minutes. After this period, the aliquots from the first two bottles in the series were transferred into HACH screw-top glass cells containing a teflon-coated magnetic stirrer bar and the turbidity in each was recorded during gentle stirring. The turbidity in each of the other bottles was measured in sequence at five minute intervals. The turbidity of each sample was plotted as a function of the administered NaOx dosing concentrations and the MSL was determined as shown in Figure 3.2. The computer program MSL [17] was used for this purpose.

3.4.2 *Metastable Limit by Coulter Multisizer*

Aliquots of 10 cm³ of filtered urine were pipetted into 12 cups specifically designed for use in Coulter multisizer analysis and were then incubated for 20 minutes at 37 °C. Thereafter aliquots were dosed pairwise at 5 minute intervals with 0.1 cm³ NaOx of increasing concentration in the same range previously described (Section 3.4.1) and were incubated for a further 30 minutes. The number of particles in each aliquot was then measured using the Coulter multisizer and plotted as a function of the administered NaOx dosing concentration. The MSL was determined as shown in Figure 3.2 using the computer program MSL [17].

3.5 Crystallization Kinetics

Many methods have been used to study CaOx crystallization [18]. These can be divided into three categories, depending on how the level of supersaturation changes during the course of the experiment [18]. Firstly, existing supersaturation may be allowed to decay. In this case, crystallization is induced and allowed to proceed without further additions and the supersaturation of the solution decreases as CaOx crystallizes. Secondly, the supersaturation may be increased; initially stable, saturated solutions in which CaOx crystallization does not occur are concentrated to further increase saturation resulting in CaOx crystallization with the aim of reproducing the way in which the kidney brings about supersaturation. In the third approach, constant supersaturation is maintained by continuously adding calcium and oxalate as crystallization occurs [18].

In the present study the supersaturation decay approach was used. The capacity of a given urine to inhibit crystal growth was quantified by monitoring the rate of growth of CaOx crystals precipitated in response to a fixed oxalate load above its MSL [16]. This was achieved by using two methods : turbidimetry and Coulter multisizer. In the former case, turbidity of the urine was recorded at 10 minute intervals for a period of 90 minutes. In the latter case, particle numbers were measured in the urine over the same time intervals. Plots of turbidity versus time and particle number versus time all showed an initial time lag followed by a steeply rising linear portion corresponding to increasing crystallization. The gradient of this section may be regarded as an index of the crystallization rate [16]. Idealised plots illustrating these two cases are shown in Figures 3.3 and 3.4.

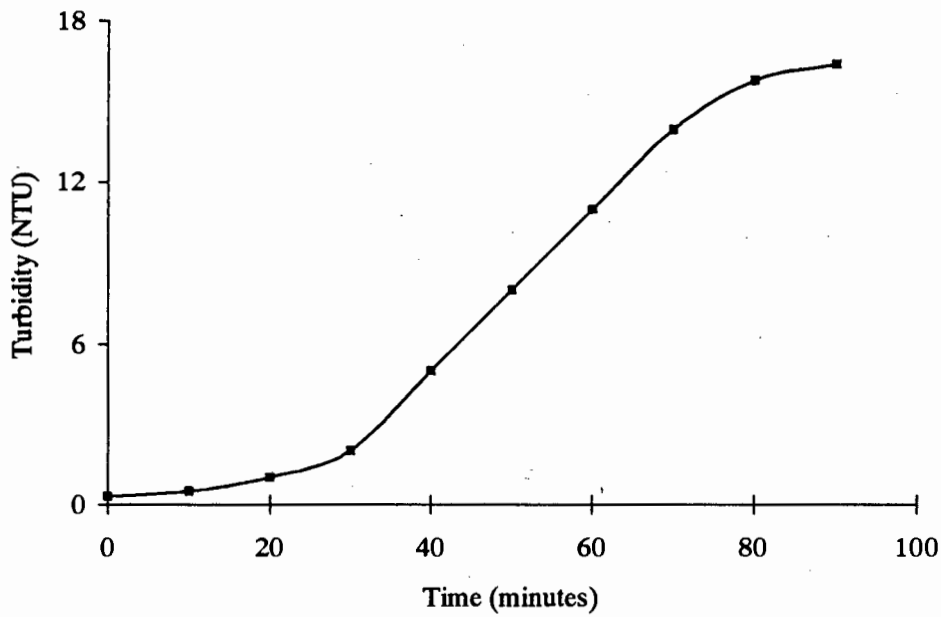


Figure 3.3 Idealised plot of turbidity as a function of time showing the initial time lag followed by a steeply rising linear portion corresponding to increasing crystallization.

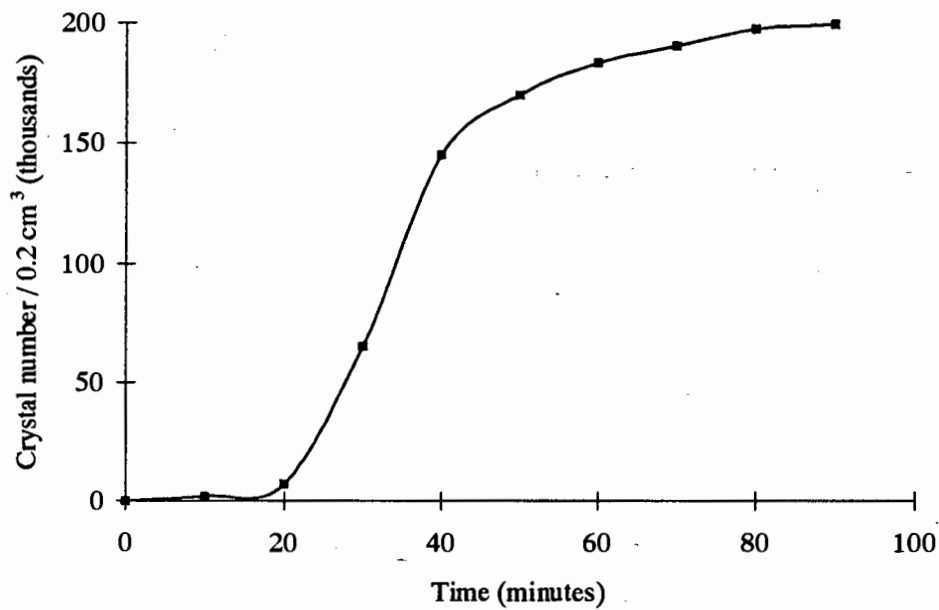


Figure 3.4 Idealised plot of particle number as a function of time showing the initial time lag followed by a steeply rising linear portion corresponding to increasing crystallization [16].

3.5.1 Crystallization Kinetics by Turbidimetry

A volume of 30 cm³ of filtered urine was poured into a clean HACH screw-top cell containing a teflon-coated magnetic stirrer bar. The cell was then incubated at 37 °C for 30 minutes. Thereafter, it was positioned in a HACH RATIO turbidimeter and the turbidity of this "blank" specimen was recorded. The reading was determined in duplicate. The sample was then dosed with 0.3 cm³ NaOx of concentration corresponding to the MSL of the control urine. Thus each urine obtained from a particular participant throughout the ascorbic acid protocol received the same NaOx challenge. Because the concentration of the administered load was "standard", the response of each urine could effectively be compared. Turbidity was determined in duplicate every 10 minutes for a period of 90 minutes.

Turbidity was then plotted as a function of time and the rate of crystallization of each urine sample was determined from the linear portion of the kinetics graph as explained earlier. In addition, samples of each urine were given NaOx challenges corresponding to the concentration just below and just above its own MSL concentrations and the rate of crystallization was then monitored as described above.

3.5.2 Crystallization Kinetics by Coulter Multisizer

Urine specimens were treated in the same way as for the turbidimetry measurements except that the starting volume was 80 cm³ and the volume of the administered NaOx challenge was 0.8 cm³. Coulter counting was done in triplicate.

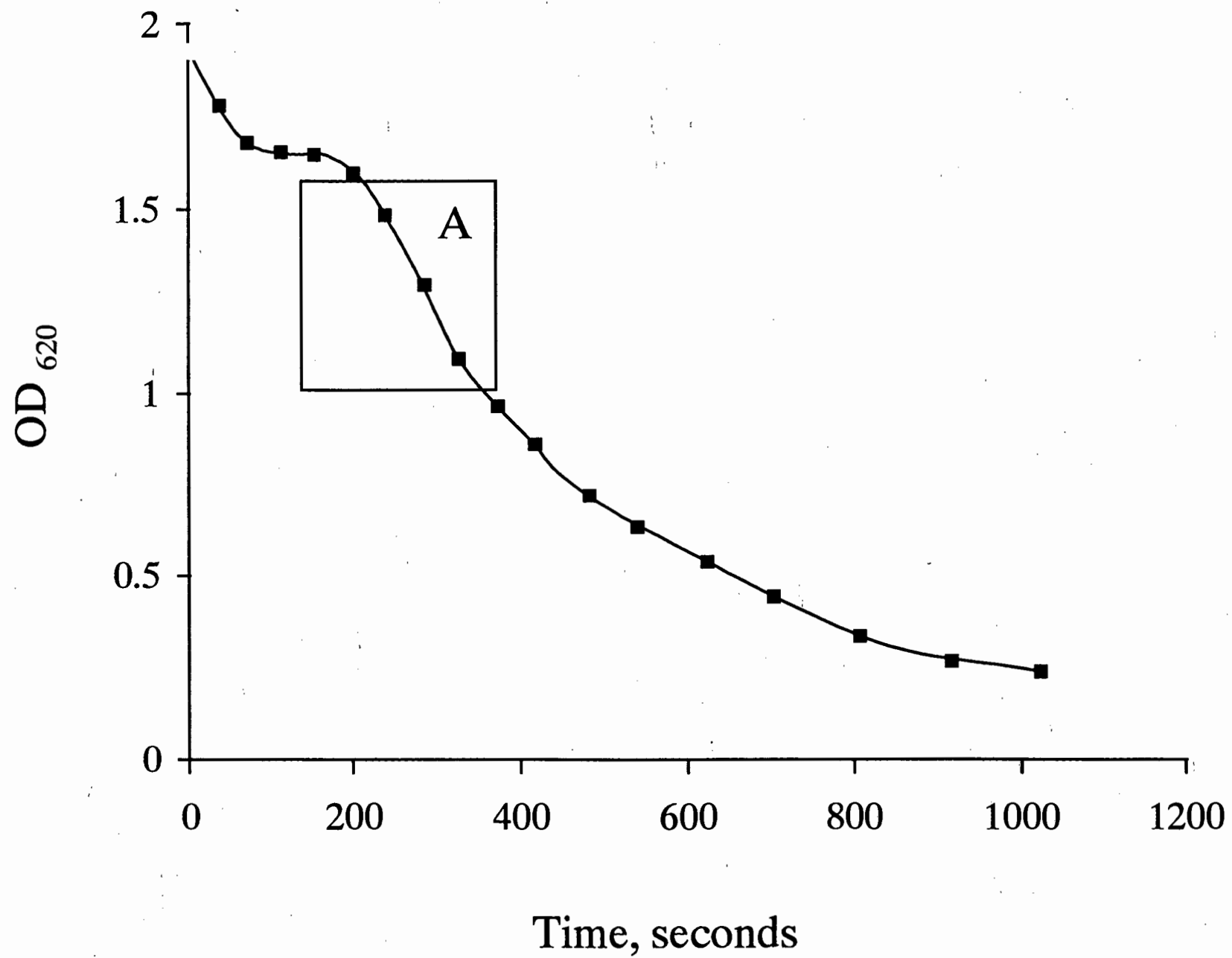
The particle count was then plotted as a function of time and the rate of crystallization determined from the slope of the linear portion of the kinetics graph as explained earlier.

3.6 Sedimentation of Calcium Oxalate in Urine

In order to simulate the calcium oxalate monohydrate (COM) crystal slurries described by Hess *et al.* [19], urine in the present study was dosed with NaOx of concentration equal to that of its MSL. Turbidity was monitored until readings remained constant, suggesting that crystal growth had ended and that the CaOx crystals in the urine were in equilibrium. This supersaturated CaOx suspension can be equated with the equilibrium slurry described by Hess *et al.* [19]. At this point the magnetic stirrer bar was removed and the sedimentation of the crystals monitored by measuring the rate at which turbidity decreased.

After this approach failed to yield meaningful results, the NaOx dosing concentration was increased to 0.2 M, thereby allowing the solution to become supersaturated more rapidly. Once equilibrium conditions had been established, 9 cm³ of the urine was transferred from the HACH RATIO screw-cap glass cuvette to a spectrophotometer tube (pathlength = 1 cm) as rapidly as possible. The tube was placed into an LKB NOVASPEC spectrophotometer and the absorbance of the supersaturated solution determined at 620 nm. Spectrophotometric measurements were determined immediately and the resultant absorbance was plotted against time. Figure 3.5 shows an idealised plot of the expected results.

Figure 3.5 *An idealised plot of OD_{620} versus time to illustrate sedimentation of the calcium oxalate crystals in a COM suspension [19]. The linear portion of the graph is annotated by (A).*



3.7 Ascorbic Acid Determination

The three urine aliquots, collected at specific intervals during the day, were initially analysed using the method developed by Tillman *et al.* 1932 [cited by 4], improved by Harris *et al.* 1933 [4] and clearly explained by Gowenlock *et al.* 1988 [20]. The method involved the use of 2,6 dichlorophenolindophenol (2,6 DCPIP) dye solution in a manual titration of the urine to which glacial acetic acid had been added. After the concentration of ascorbic acid had been determined in this way, the sample was analysed by flow-injection analysis. (Chapter 4). Where the ascorbic acid concentration exceeded $150 \mu\text{g}\cdot\text{cm}^{-3}$, the urine was diluted with acetic acid (10 cm^3 glacial acetic acid in 100 cm^3 distilled water):

3.7.1 Titrimetric Determination of Ascorbic Acid with 2,6 DCPIP

The technique is based on a redox titration of ascorbic acid with 2,6 DCPIP in an acidic medium. The dye is reduced to a colourless leucobase (Figure 3.6) while the ascorbate is oxidised to dehydroascorbate [20]. If the latter is present, it must first be reduced to ascorbate before it can be measured.

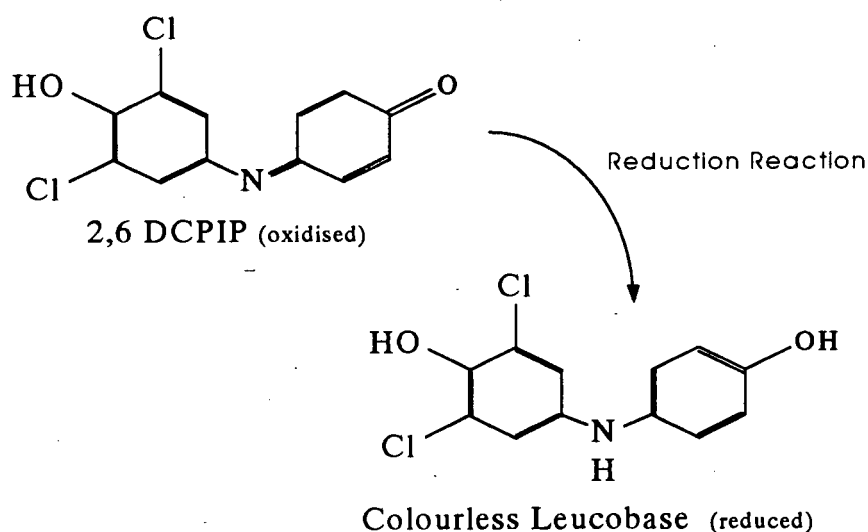


Figure 3.6 Chemical formulae of 2,6 DCPIP and the reduced colourless leucobase.

3.7.2 Preparation of 2,6 DCPIP Dye and Ascorbic Acid Standards

A quantity of dye powder (100 mg) was accurately weighed and dissolved in 250 cm³ distilled water. A fresh solution was prepared every two to three days. The dye solution was standardised against a 20 µg.cm⁻³ ascorbic acid solution. This standard solution was prepared in the following manner. Acetic acid was prepared in the ratio of 10 cm³ glacial acetic acid in 100 cm³ distilled water. A 400 µg.cm⁻³ ascorbic acid stock solution was prepared by weighing out 40 mg pure ascorbic acid into a 100 cm³ volumetric flask. The flask was made up to volume with the prepared acetic acid solution. The 400 µg.cm⁻³ ascorbic acid stock solution was diluted with acetic acid to make up a 20 µg.cm⁻³ standard (2.5 cm³ stock solution in 50 cm³ acetic acid), a 60 µg.cm⁻³ standard (7.5 cm³ stock solution in 50 cm³ acetic acid) and a 100 µg.cm⁻³ standard (12.5 cm³ stock solution in 50 cm³ acetic acid). The 20 µg.cm⁻³ ascorbic acid standard was then titrated against 0.5 cm³ of the blue dye solution. Since 1 cm³ of 2,6 DCPIP is equivalent to 0.2 mg of ascorbate, the expected volume of the titrant is 5 cm³ [20].

3.7.3 Technique for the Titration of Urine Samples

As stated previously, 45 cm³ of fresh urine was pipetted by each participant into a small glass bottle containing 5 cm³ glacial acetic acid at three specified times during the 24 hour collection period. The samples were analysed within a few hours of collection.

A volume of 0.5 cm³ 2,6 DCPIP dye solution was pipetted into a glass vial using a 0.1 cm³ GILSON micropipette. The mixture of 9 parts urine to 1 part glacial acetic acid was placed in a 10 cm³ burette, graduated at 0.2 cm³ intervals, and titrated directly with the dye. The dark blue dye solution turned a brilliant red as the first drop of the acidified urine came into contact with it. As the endpoint of the titration was approached, the solution became a paler pink colour, the endpoint being attained when the red colour had been completely discharged. The volume of the urine solution required to discharge the red colour of the acidified dye solution was then recorded.

Equation 3.1 allows for the determination of the concentration of ascorbate present in the urine sample.

$$\text{Urinary ascorbate } (\mu\text{g.cm}^{-3}) = 111 / \text{urine vol (cm}^3\text{)} \quad \text{Equation 3.1}$$

3.8 Tiselius Risk Index

The Tiselius risk index expresses the biochemical risk of calcium oxalate stone formation [21]. The mathematical expression is given by Equation 3.2.

$$(\text{Ca} / \text{Cr})^{0.71} \times (\text{Ox} / \text{Cit}) / (\text{Mg} / \text{Cr})^{0.14} \times (\text{Cit} / \text{Cr})^{0.10} \quad \text{Equation 3.2}$$

where Ca, Ox, Mg, and Cit are the excretions (mmol per 24 hour) of calcium, oxalate, magnesium and citrate respectively. Creatinine concentrations are expressed in mols per 24 hours. The higher the value the greater the risk.

3.9 Calcium Oxalate Relative Supersaturation

Calculation of relative supersaturation for CaOx was undertaken using the computer program EQUIL [22]. Each of the chemical components of the urine was included in the calculation. Samples in which ascorbic acid interference prevented urate concentration from being determined, CaOx relative supersaturation values were calculated using a urate concentration of zero.

3.10 Scanning Electron Microscopy

Approximately 10 cm³ raw urine and urine preserved with EDTA was poured into a clean glass centrifuge tube and centrifuged at 4000 rpm for 20 minutes using a HERAEUS SEPATECH LABOFUGE 200. The sedimented samples were then aspirated carefully using a clean pasteur pipette and filtered through a 0.22 µm Sartorius filter paper placed in a Sartorius membrane filter clamp. Thereafter the filter paper with the deposited crystals was pasted onto an aluminium stub. These stubs were then coated with approximately 100 nm of Au/Pd in a BLAZER'S vacuum coater. Specimens were examined using a LEICA CAMBRIDGE S440 sigma scanning electron microscope operating at an acceleration voltage of 10 kV and a probe current of 50 pA.

3.11 Statistical Analysis

Statistical analysis employed a repeated measures or split plot design [23]. The layout for the design for statistical analysis of the 10 participants is shown in Table 3.1 overleaf.

The analysis of variance, in the split plot design, falls into two parts, the whole plot analysis and the subplot analysis. The data for each fraction for each participant comprises the whole plot data. Thus the values $a_{11}, a_{12}, \dots, a_{18}$ comprise the data for one whole plot with total A_1 . The totals $A_1, B_1, C_1, \dots, A_{10}, B_{10}, C_{10}$ comprise the whole plot total and give rise to the whole plot analysis. The variation between these totals is due to variation between participants and to variation between fractions, these being the factors we can identify for the whole plots. The remaining variation between the whole plot totals comprise random or error variation; specifically whole plot error. The subplots are the values $a_{11}, a_{12}, \dots, a_{18}$ etc. within each whole plot. These values, over all whole plots, gives rise to the subplot analysis. Part of the variation between the subplots is the interaction between fractions and samples. The remaining variation is considered error variation, and this is referred to as subplot error.

Table 3.1 *Summary of the method for the statistical analysis employed in this study.*

| Sample No. | Participants | | | | | | | | | | | |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-------|--|--|-------------------|-------------------|-------------------|
| | 1 | | | 2 | | | | | | 10 | | |
| | Fraction | | | Fraction | | | | | | Fraction | | |
| 1 | a ₁₁ | b ₁₁ | c ₁₁ | a ₂₁ | b ₂₁ | c ₂₁ | | | | a _{10,1} | b _{10,1} | c _{10,1} |
| 2 | a ₁₂ | b ₁₂ | c ₁₂ | a ₂₂ | b ₂₂ | c ₂₂ | | | | a _{10,2} | b _{10,2} | c _{10,2} |
| . | . | . | . | . | . | . | | | | . | . | . |
| . | . | . | . | . | . | . | | | | . | . | . |
| 8 | a ₁₈ | b ₁₈ | c ₁₈ | a ₂₈ | b ₂₈ | c ₂₈ | | | | a _{10,8} | b _{10,8} | c _{10,8} |
| Totals | A ₁ | B ₁ | C ₁ | A ₂ | B ₂ | C ₂ | | | | A ₁₀ | B ₁₀ | C ₁₀ |

fraction : indicates the manner in which the urine was stored when collected (i.e. a = bulk urine sample, b = representative sample + EDTA and c = representative sample - EDTA)

sample no. : indicates the number of the sample in the order it was collected during the protocol (1, 2 : before ascorbic acid protocol; 3, 4, 5 : during ascorbic acid protocol; 6, 7, 8 : after ascorbic acid protocol)

A feature of this design is the presence of two sources of error variation. Thus standard errors of the fractions are based on the whole plot error and the standard errors of the sample and the interaction means are based on the subplot error.

3.12 References

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Chapter 4.

Flow-Injection Analysis

4.1 Introduction and Theory

Solution analysis in any chemical laboratory requires a number of operations which include solution handling, analyte detection, data collection and the computation of results. The latter two tasks can be carried out easily with the availability of sophisticated computers and detectors. However, a knowledge of several skills is required in solution handling, such as decanting, pipetting, mixing and other volumetric operations. These skills are still performed manually, even in advanced chemical laboratories, using apparatus that was designed two centuries ago. Recent trends have been to move away from these large volume manipulations to smaller volumes. Smaller volumes are economically and environmentally favoured.

The move to small volumes introduces the concept of "flow operations". The mechanical handling of volumetric vessels is replaced by handling micro-volumes of solution in closed, small-bore tubes. Flow operations facilitate control in time and space since evaporation does not occur. The paths along which the solutions flow are exactly repeatable, with the result that the solution mixing and formation of reaction products is highly reproducible. In the case of solution spectrophotometry, the standard batch-method detectors are readily equipped with flow-through cuvettes (flowcells), to monitor coloured reaction products.

Flow-injection analysis (FIA) [1, 2] has emerged in the last decade as a very powerful solution handling and data gathering technique. FIA is based on controlled flow operations. It is used in many analytical and research laboratories world-wide. FIA can be applied to many existing wet-chemical methods and is an ideal means to automate such analyses.

FIA belongs to a group of methods involving the injection of a liquid sample into a non-segmented, continuous carrier stream of suitable liquid. This group of methods comprises techniques including chromatography, electrophoresis and field flow fractionation. These

different techniques vary in one fundamental aspect - the function of the modulator that alters the original square-wave input provided by the sample injection, into a chromatogram, electrophogram, fractogram or fiagram respectively, as depicted in Figure 4.1. The modulator, or force that occurs within it, is a column for chromatography, an electric field for electrophoresis, an external force for field flow fractionation, and a reaction coil for flow-injection analysis.

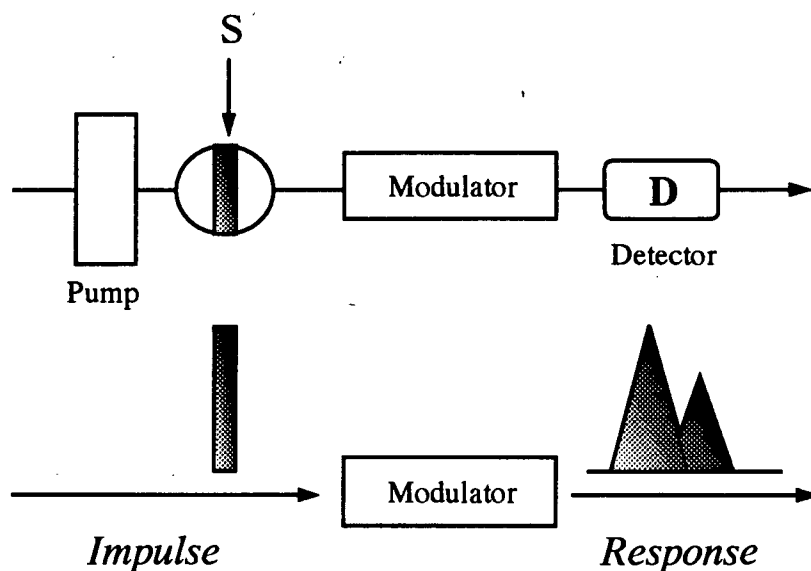


Figure 4.1 *Flow scheme (top) and concept (below) of analytical techniques based on the injection of the sample analyte into a flowing carrier stream [1]*

4.1.1 The FIA manifold

The simplest flow-injection analyser consists essentially of four components: (1) a pump which is used to propel the carrier stream through a narrow tube, (2) an injection port (valve), by means of which a well-defined volume of sample solution is injected into the carrier stream in a reproducible manner, (3) a reactor in which the sample zone disperses and reacts with the components of the carrier stream, and (4) a detector that continuously monitors some physical parameter (such as absorbance, electrode potential, chemiluminescence, pH, etc.) of the carrier stream as it passes through the flowcell. The shape and magnitude of the resulting transient

signal reflects the concentration of the injected analyte, along with thermodynamic and kinetic information of the processes occurring within the flow stream. Together, the complete FIA system is termed a manifold.

The pump is most often a multi-roller peristaltic pump, accommodating one or more pump channels. By variation of individual pump tubing diameters, equal or different volumetric pumping rates may be obtained. Pulsing of the flow stream is minimised if pump tubing comes into contact with as many of the pump-rollers as possible.

The injection method used may be one of four types : syringe injection, valve injection, hydrodynamic injection and time-based injection. The method of injection in this study was by means of a 6-port, rotary injection valve. This valve has two stages of operation, (1) "load" and (2) "inject". In the "load" position, the sample loop is filled with sample whilst the carrier is shunted past the valve, via a bypass, to prevent pressure build up [3]. The valve is switched to the "inject" position with the result that the sample zone is injected into the carrier stream and swept towards the detector in the flowing carrier stream.

The reactor is commonly made of plastic tubing (usually 0.5 - 1.0 mm i.d.^{*}) which can be coiled, knitted or knotted [4]. Selection of the reactor is naturally dependent on the chemistry of the system. The geometric deformation of the flow path of the sample has an effect on the radial and axial mixing of the sample with the carrier stream components. Many other reactors, such as immobilised enzymes, ion exchangers, and gas diffusion membranes, can be used.

The final component in a FIA manifold is the detector, and closely associated, the signal recording device. Amongst the first methods adapted for FIA were colorimetric methods for ammonia and phosphate, where spectrophotometers were readily available. A flow cell allows such detection systems to be readily employed in flow-injection analysis without further adaptation or capital outlay. The signal recorder (often a chart recorder) is essential in a FIA system since it records the detector response from which the result may be determined. The

* i.d. represents the inner diameter of the tubing.

response profile enables the monitoring of the systems operational status (e.g. monitoring any baseline drift).

4.1.2 Quantitation in FIA

Analysis by means of FIA is unique. The manifold shown in Figure 4.2 is the most commonly used single-line manifold. The pump, P, propels the carrier stream (C) to the point at which the sample, S, is injected into the carrier stream (C) which contains the reagent. Dispersion of the injected sample zone occurs and a reaction takes place. The duration of the reaction is controlled by the length of the reaction coil, RC, and the flow rate, F. The analytical signal is determined by means of a detector and the resultant signal recorded. Typical recorder output has the form of a peak (Figure 4.2), the height (H), width (W) and area (A) of which may be related to the analyte concentration.

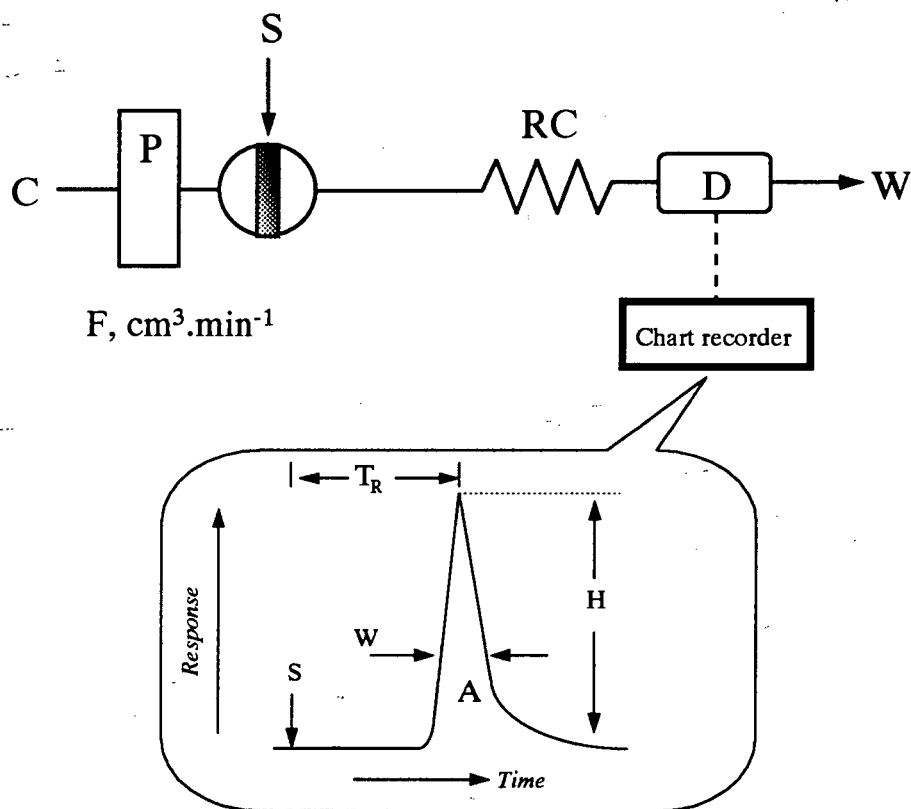


Figure 4.2 Scheme of the most commonly used single-line manifold shown with the typical signal response obtained.

The time span from sample injection to peak maximum, is the residence time (T_R) during which the reaction takes place. Well-designed FIA systems have a short residence time of typically 5 to 20 seconds and thus a sample cycle less than 30 seconds. The injection volume is usually in the range of 10 - 200 μl , with no more than 0.5 cm^3 of sample required to flush the injection loop between sample cycles. This makes FIA a very economical technique.

A variation of FIA is "Reversed FIA" (rFIA) [1]. This technique is based on the injection of the reagent into a carrier stream of the sample. This approach is common when there is an abundance of sample (e.g. analysis of a sea-water sample), or when the reagent is very expensive or hazardous. Reversed flow-injection analysis is used in this study, however, theoretical and quantitation aspects of flow-based technology apply both to FIA and rFIA.

4.1.3 Dispersion and Mixing Processes

FIA is based on three principles : sample injection, controlled dispersion of the injected sample zone and reproducible timing. In contrast to all other techniques, chemical reactions in FIA are taking place while the sample reagent is dispersing within the reagent (i.e. while the concentration gradient of the sample zone is being formed by dispersion processes). The concept of dispersion, controlled within space and time is the foundation-stone of FIA.

The parameters which characterise the transient signal define the dispersion. The sensitivity[†] of the response is one of the parameters which is a characteristic of the transient signal. Sensitivity is influenced by the physical parameters of the manifold such as the flow rate, reaction coil length, sample injection volume and the inner diameter of manifold tubing. As a result, dispersion is dependent on these same physical parameters [1].

The sample solution within the cavity of the injection valve prior to injection is homogenous and has an original concentration C^0 . If this sample solution was scanned, it would yield a square-wave signal, the height of which would be proportional to the sample concentration (Figure 4.3). When the sample zone is injected into a moving carrier stream the

[†] the response per unit concentration

resultant dispersion depends, amongst others, on the geometry of the reactor and the flow rate. The signal is a response curve which has a peak shape that reflects a continuum of concentrations, forming a concentration gradient within which no single element has the same concentration of sample material as a neighbouring one (Figure 4.3). As a result of dispersion, the maximum peak height (C^{\max}) will not exceed that of the theoretical square-wave response. A measure of the extent of dispersion is termed the "Dispersion Coefficient" (D).

Dispersion in FIA has been defined as the ratio of the original analyte concentration, C^o , to the concentration of the analyte in the element of fluid which corresponds to the maximum of the peak, C^{\max} [5] as shown in Equation 4.1.

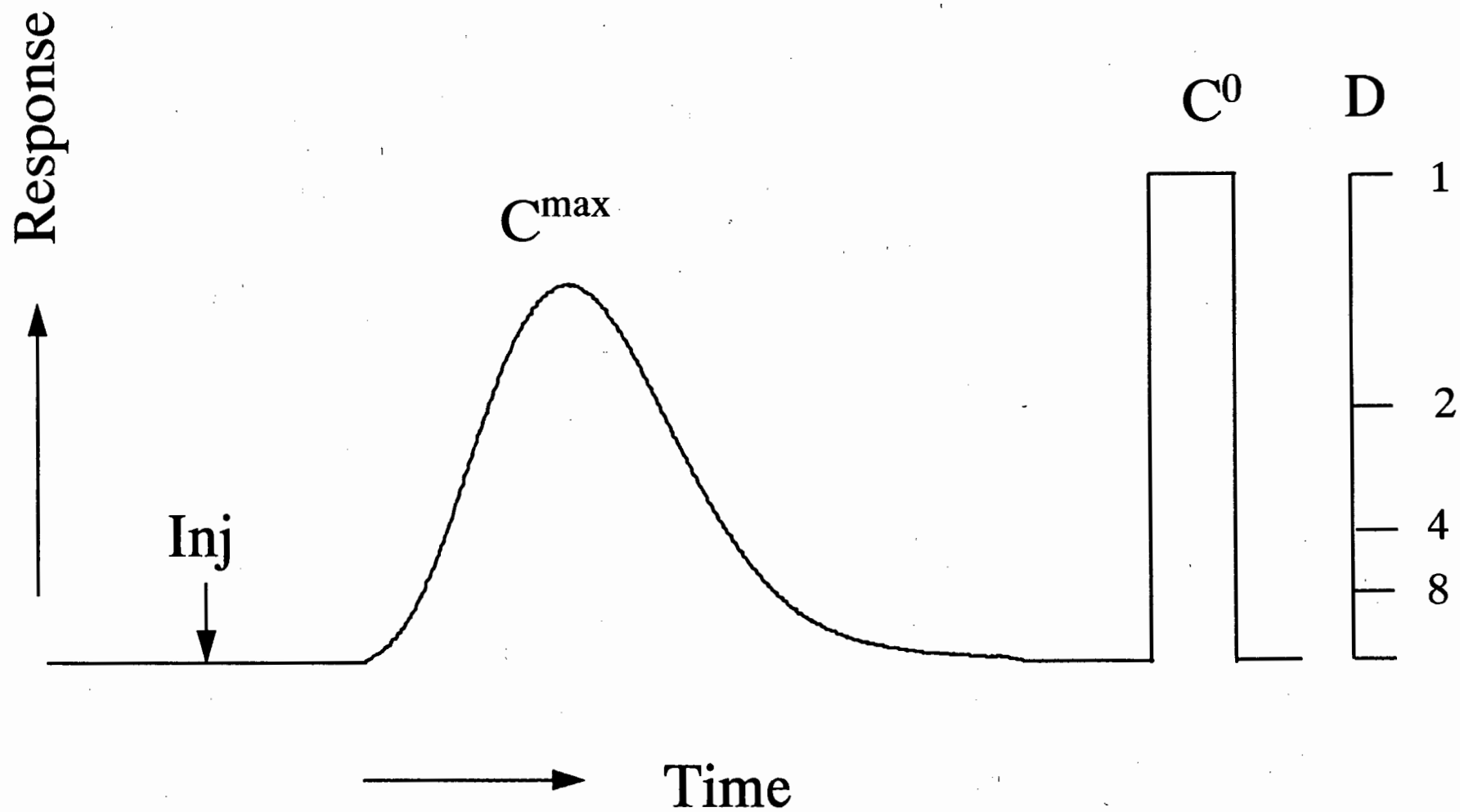
$$D = C^o / C^{\max} \quad \text{Equation 4.1}$$

If the analytical response is based on the usual peak height measurement and provided Beer's Law holds, the dispersion equation can be written as Equation 4.2.

$$D = C^o / C^{\max} = H^o / H^{\max} \quad \text{Equation 4.2}$$

Dispersion is classified as limited ($D = 1$ to 3), medium ($D = 3$ to 10) and large ($D > 10$). If one or several reactions resulting in colour formation is to occur, a medium dispersion of the sample zone is required so that the centre of the sample zone is effectively mixed with the carrier stream [3]. It is necessary to find a suitable compromise between mixing and reaction time on one hand, and keeping zone broadening of the peak to a minimum on the other [3].

Figure 4.3 *Dispersion within a FIA manifold. Prior to injection the sample zone (top right) is undiluted and has a concentration C^0 . After injection the zone disperses, changing the original square-wave form (bottom right) to yield a continuous concentration gradient (centre) with maximum concentration C^{max} at the peak apex. The dispersion coefficient, D , scale is shown calculated relative to the original square-wave form.*



Dispersion for a manifold can be computed using a simple experiment described by Ruzicka and Hansen in an excellent monograph on FIA [1]. The reagents employed in the experiment consist of a 0.02 N borax carrier solution (3.814 g sodium tetraborate decahydrate dissolved in 1 dm³ of glass distilled water) and a bromothymol blue dye solution. A bromothymol blue stock solution was prepared by dissolving 0.400 g bromothymol blue in 25 cm³ 96 % ethanol. This solution was then made up to 100 cm³ with the borax carrier solution. A working solution of bromothymol blue was prepared by 100-fold dilution of the stock solution.

The simplest way to measure dispersion is to inject a well-defined volume of bromothymol blue dye into a colourless carrier stream and continuously monitor the absorbance with a spectrophotometer at 620 nm. The height of the recorded peak (H^{\max}) is measured and compared to the distance between the baseline and the signal recorded when the flowcell is filled with the undiluted bromothymol blue dye (H^0). Provided Beer's Law holds, the dispersion coefficient can be calculated from Equation 4.2. The manifold may be adapted in such a way as to control the extent and nature of the dispersion. Dispersion for the optimised manifold used in this study was 3.0, and is classified as medium dispersion.

Mixing processes that take place in FIA comprise convective transport, secondary flow, molecular diffusion, and turbulent flow when merging streams. Convective transport in a straight tube results in a parabolic velocity profile with the fluid at the centre of the tube having twice the average velocity than the fluid at the sides of the tube. Secondary flow arises when curvature of the tubing (such as coiling) results in the laminar flow conditions being disrupted. This type of flow promotes mixing of the sample zone with the carrier stream.

Molecular diffusion occurs when concentration gradients are established within the fluid under the effects of laminar and secondary flow. Molecular diffusion is the spontaneous motion of particles from a region of high concentration to a region of low concentration in an attempt to uniformly distribute all molecules. Axial diffusion occurs in a direction parallel to the tube walls, and radial diffusion in a direction orthogonal to the tube walls. Both types of diffusion further aid mixing. Finally, turbulent flow arises when the flow streams are disturbed

by forces other than those previously mentioned, such as merging streams, stirred mixing chambers, or mini-columns.

With a knowledge of the underlying theory of FIA, preliminary investigations toward the establishment of a FIA method for the determination of ascorbic acid were made.

4.2 Preliminary Investigations

Attempts were made to establish a FIA method using chloramine-T and a starch-potassium iodide (KI) indicator as described by Lazaro *et al.* [6, 7] (Chapter 2). The system was set up using the reported parameters, namely, [chloramine-T] = $5 \times 10^{-4} \text{ mol.dm}^{-3}$; starch-KI indicator = 5.25 g soluble starch and 0.50 g KI in 500 cm^3 of distilled water; flow rate = $2.0 \text{ cm}^3.\text{min}^{-1}$; injection volume = $96 \text{ }\mu\text{l}$; reactor length = 55 cm; inner diameter of reactor = 0.5 mm; [sulphuric acid] = 0.90 M; and analytical wavelength = 650 nm.

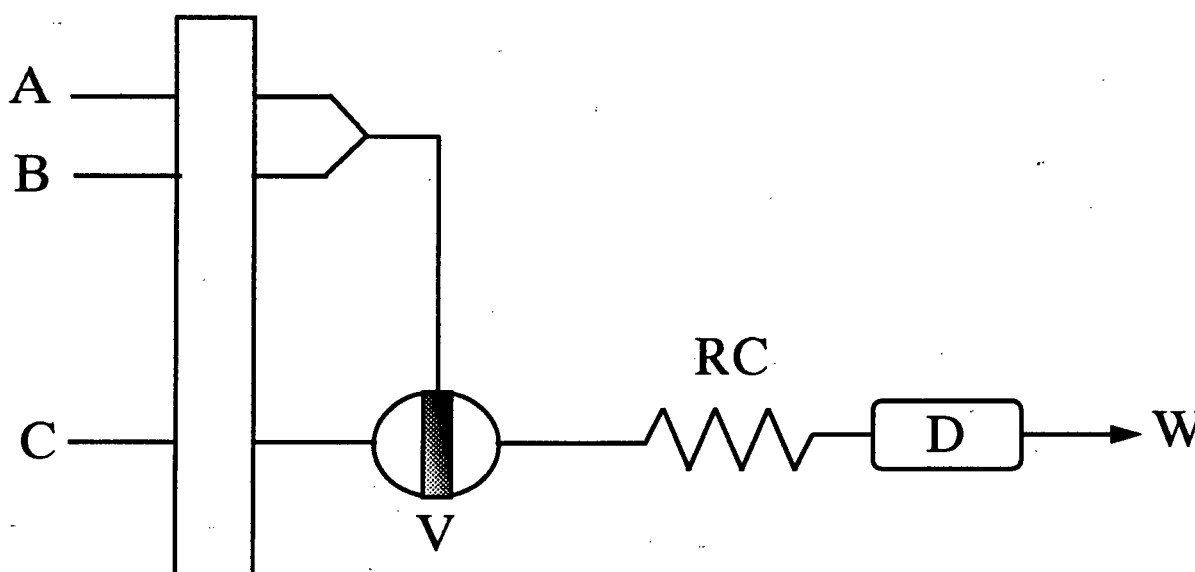


Figure 4.4 Manifold used in the determination of ascorbic acid in urine by the starch-KI and chloramine-T [6] method. A = starch-KI, B = Sample, C = chloramine-T, V = Injection valve, RC = reaction coil, D = detector, and W = waste.

Figure 4.4 shows the manifold used by these investigators. The repeatability and reproducibility using the parameters given were very poor. Following this, each of the experimental parameters (such as concentration, flow rates, injection volume) were examined. Despite extensive effort to find suitable settings of these parameters for the determination of ascorbic acid, the repeatability and reproducibility remained unsatisfactory. Further attempts to reproduce the FIA system described by Lazaro *et al.* [6, 7] were abandoned. An alternate FIA system was designed and developed to determine ascorbic acid in urine.

4.3 The Determination of Ascorbic Acid

A popular method for the determination of ascorbic acid is the titration using 2,6 DCPIP [8] (Section 2.5.1). An objective of this study was to automate the 2,6 DCPIP titration using FIA. The chemistry used in the flow-injection manifold is identical to that employed in the manual titration of ascorbic acid using 2,6 DCPIP. The application of FIA to this manual titration has not been previously been attempted.

As discussed previously (Section 2.5.1), the endpoint of this reaction is colourless. After preliminary experiments it was decided to using a point other than the colourless end point to determine ascorbic acid. This point is referred to as a "pseudo-end point". Although a spectrophotometer may be used to monitor disappearance of a colour, the use of a FIA titration manifold with measurement of the peak width [1] was undesirable. A measure of the peak height for ascorbic acid quantification was preferred.

A FIA manifold for the determination of ascorbic acid using a wavelength in the visible region of the absorption spectrum was used. Although some activity is observed in the UV region (less than 360 nm), detection in this study is limited to the visible region (Figure 4.5). The selection of the pseudo-end point was crucial and careful consideration of accuracy and precision was made.

4.3.1 *Optimisation of the FIA Manifold*

The aim of optimisation of a manifold is to obtain adequate sensitivity for the analysis with high sample throughput, minimum peak widths (and hence peak-tailing) and good precision and accuracy. The manifold configuration was optimised by varying physical (analytical wavelength, flow rate, injection volume and reaction coil length) and chemical (concentrations of the reagent and carrier solutions) parameters of the manifold until a setting satisfying the requirements above was found.

A common approach is to investigate the effect of one parameter at a time until an optimum is attained. The next variable is then optimised. Provided minimal interaction between variables exists, this method gives an optimised set of conditions without too many experiments. The requirement of the method to operate suitably and accurately, not necessarily at the optimum sensitivity, was kept in mind throughout the optimisation process.

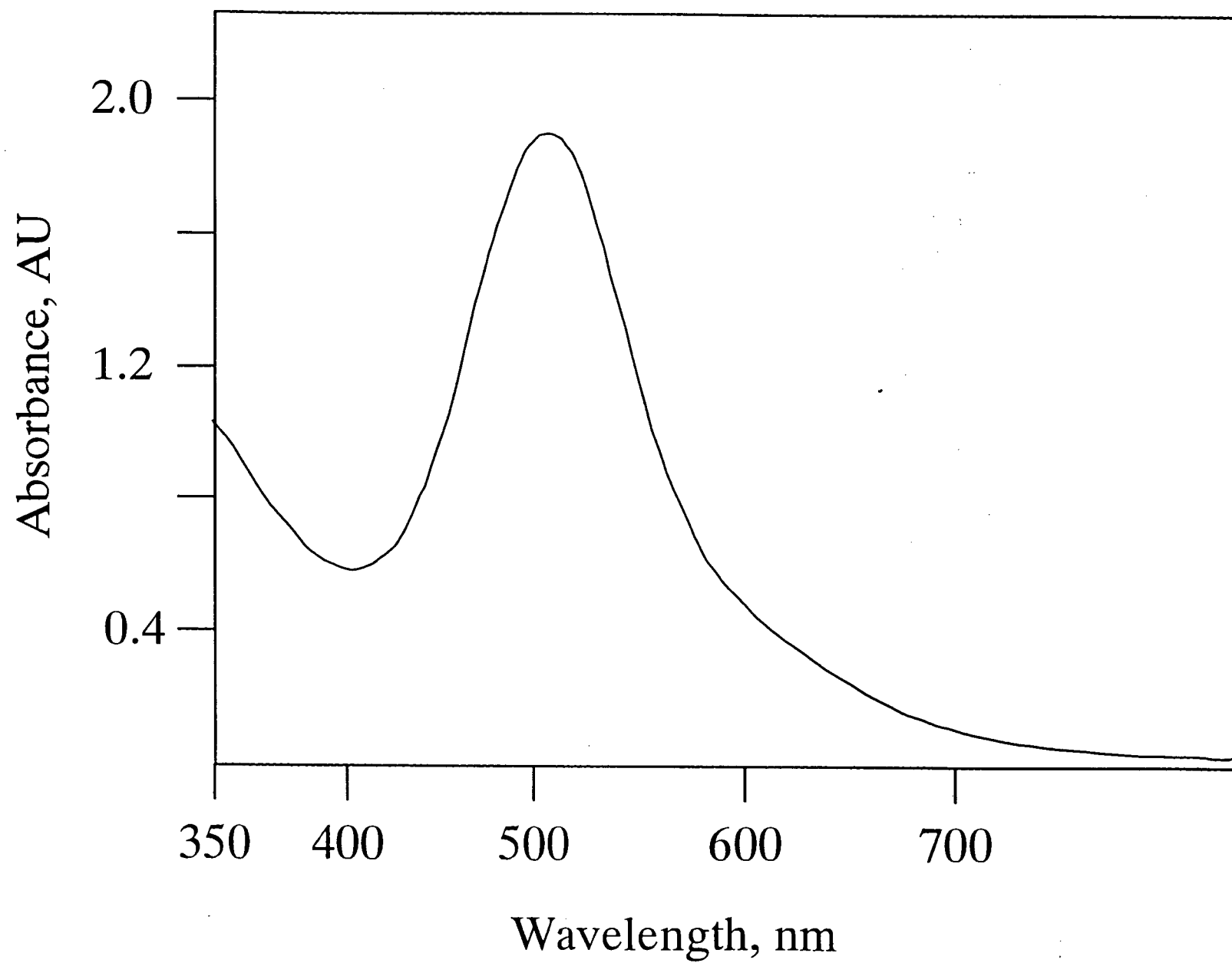
4.3.1.1 Analytical Wavelength

To realise maximum sensitivity, spectrophotometric absorption measurements are made at the wavelength corresponding to an absorption maximum since the change in absorbance per unit concentration is greatest in this region [9]. It is preferable to avoid determinations which correspond to the steep slopes of an absorption curve since minor imprecision in wavelength resettability incur large errors. Selection of an analytical wavelength was made by scanning the absorption spectrum of the absorbing species in the range 350 nm to 650 nm using a PU 8700 SERIES UV/visible scanning spectrophotometer.

The absorbance maximum is found at 518 nm (Figure 4.5). Changes in dye (2,6 DCPIP) concentrations did not alter the position of this absorbance maximum. Furthermore, the maximum did not shift when either ascorbic acid standards or urine samples were analysed.

Consideration of the wavelength maximum is important since any change thereof (e.g. when using different experimental conditions during an optimisation) would affect the resultant response by giving a lower response than the true response.

Figure 4.5 *Diagram showing the maximum absorbance peak at 518 nm for a 40 $\mu\text{g}\cdot\text{cm}^{-3}$ ascorbic acid standard solution using a scanning UV-visible spectrophotometer.*



4.3.1.2 Flow Rate

The flow rate of the carrier stream affects the dispersion and mixing of the reagent zone with the components of the carrier stream (the sample in rFLA). The regulation of this dispersion and mixing may be partially controlled by the flow rate. Low flow rates (less than $0.5 \text{ cm}^3 \cdot \text{min}^{-1}$) cause peak broadening and very high flow rates (greater than $3 \text{ cm}^3 \cdot \text{min}^{-1}$) often result in poor reproducibility. A compromise is essential.

The careful selection of flow rate is of particular importance in this analysis since the residence time (T_R) of the species is crucial. A T_R that is too long will result in the reaction going to completion, and a T_R that is too short does not permit sufficient mixing between the sample and reagent components. In cases where it is desirable to increase the T_R , it should be noted that it is preferable to increase the reaction coil length (Section 4.3.1.3) rather than decreasing the flow rate [3].

Factors considered in the optimisation of the flow rate include sensitivity (calibration slope) and linearity of this calibration (correlation coefficient). RSD of replicate injections (defined here as the precision) is also considered, as is the extent of peak tailing (closely related to the peak width). These results are shown in summary in Table 4.1.

An increase in the flow rate results in (a) increased sensitivity; (b) a decreased peak width (and hence less peak tailing and sample carry-over), and (c) an increased sampling (injection) rate as a result of (b). A flow rate of $1.99 \text{ cm}^3 \cdot \text{min}^{-1}$ was selected for this work based on the high sensitivity, a low RSD for replicate injections, economy of sample and reagent solutions, minimal carry-over, little peak tailing and an accompanying high sampling rate obtained for at this flow rate.

Table 4.1 *Factors considered in the optimisation of the flow rate.*

| Flow Rate (cm ³ .min ⁻¹) | Absorbance (AU) ^a | RSD (%) ^b | Peak Width (mm) ^c | Slope (x 1000) (AU/μg.cm ⁻³) | Corr. Coeff. (r) |
|--|---------------------------------|-------------------------|---------------------------------|--|------------------------|
| 0.74 | 0.222 | 0.87 | 8.1 | - 2.22 | 0.9980 |
| 1.13 | 0.269 | 0.68 | 5.0 | - 2.33 | 0.9997 |
| 1.53 | 0.256 | 1.89 | 4.2 | - 2.36 | 0.9988 |
| 1.99 | 0.287 | 0.37 | 3.1 | - 2.57 | 0.9991 |
| 2.41 | 0.284 | 0.78 | 2.7 | - 2.70 | 0.9890 |
| 2.73 | 0.294 | 1.23 | 2.5 | - 2.65 | 0.9983 |

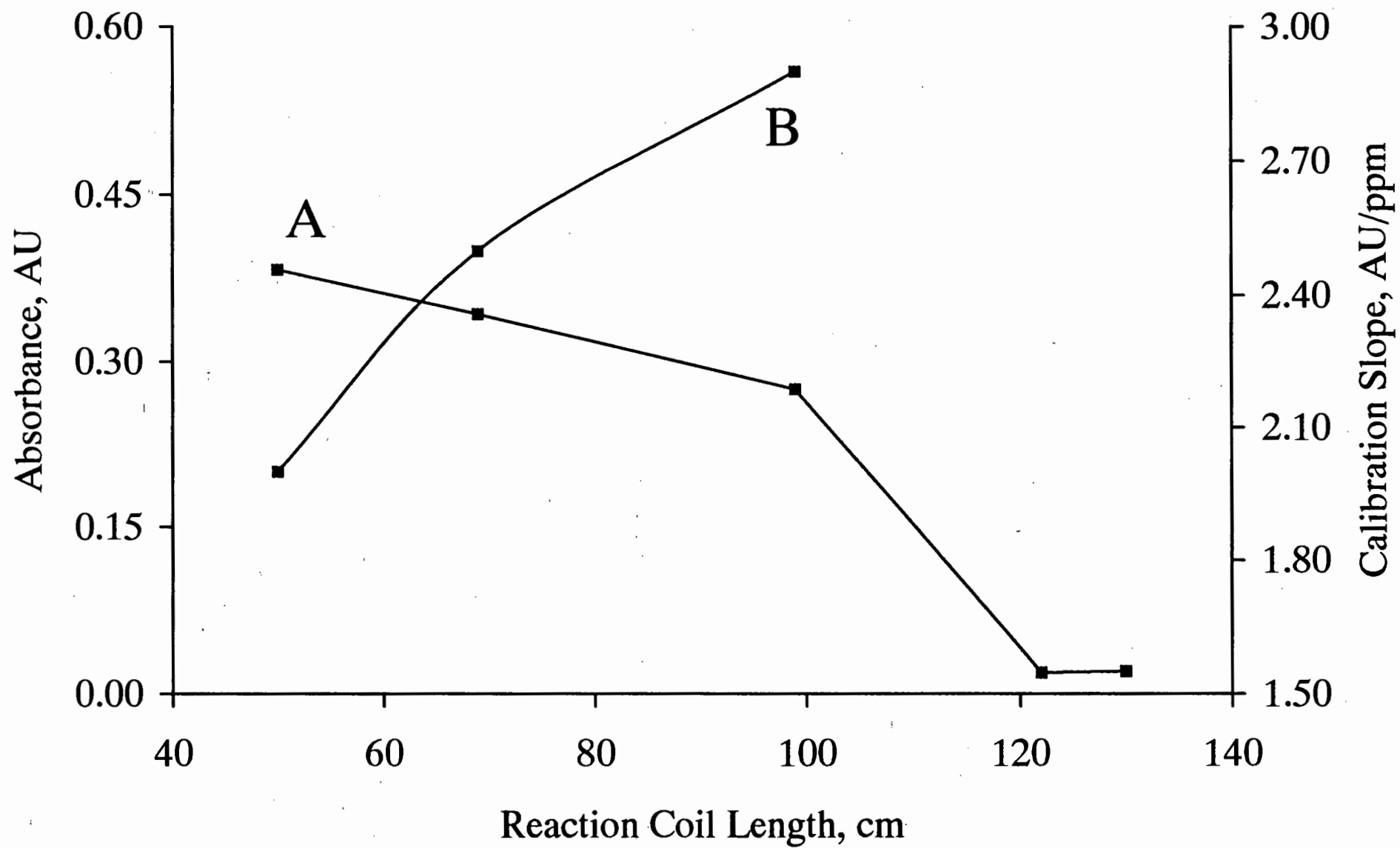
^a Absorbance for 40 μg.cm⁻³ ascorbic acid^b RSD for 5 replicate injections^c Peak width measured at 50 % of the peak height

4.3.1.3 Reaction Coil Length

A reaction coil length that yielded good reproducibility of response, provided an adequate, though not too long, residence time, and kept peak tailing to a minimum is essential. In addition, a suitable residence time is required to measure the response at a highly reproducible stage of the reaction between 2,6 DCPIP and ascorbic acid. The residence time may be conveniently changed at a fixed flow rate by varying the reaction coil length.

Figure 4.6 shows the effect of the reaction coil length on the calibration slope and sensitivity of the reaction.

Figure 4.6 *A plot of absorbance of (A) a $40 \mu\text{g.cm}^{-3}$ ascorbic acid solution and (B) slope of the calibration as a function of reaction coil length.*



Although maximum sensitivity was observed when using a 50 cm reaction coil, the reproducibility was poor due to a very short residence time and insufficient mixing of the sample zone. At the other extreme, reaction coil lengths longer than 100 cm resulted in too long a residence time. The reaction went to completion (colourless) and no response was obtained.

Consideration of the calibration slope and concentration range is important. Too small a difference between the response of differing ascorbic acid standard concentrations is undesirable, as is a very limited concentration range. Figure 4.7 shows an increasing calibration slope as the reaction coil length increases to a maximum of 100 cm. The response is negligible beyond this length, due to reaction completion. The concentration range for a reaction coil length of 100 cm was $150 \mu\text{g}\cdot\text{cm}^{-3}$. A 100 cm coil was selected as the optimum length for the reaction coil of the system. The reproducibility for replicate injections ($n = 5$) when using the 100 cm reaction coil length was better than 2 % for a $40 \mu\text{g}\cdot\text{cm}^{-3}$ ascorbic acid standard.

4.3.1.4 Reagent Injection Volume

A schematic diagram of the injection valve used in this study (Figure 4.7) shows the pump drawing up the reagent (2,6 DCPIP) into the injection loop - the "load" position, while the carrier (ascorbic acid standard or urine sample) bypasses the loop. The valve is then switched to the "inject" position and the reagent is injected into the urine carrier stream as it passes through the loop. Once the maximum absorbance has been reached after the injection, the valve is returned to the "load" position.

A "wash" solution of 50 % nitric acid, followed by distilled water, was used to flush the manifold and injection loop between samples. This "wash" prevented build-up of particulate matter on the walls of the manifold tubing.

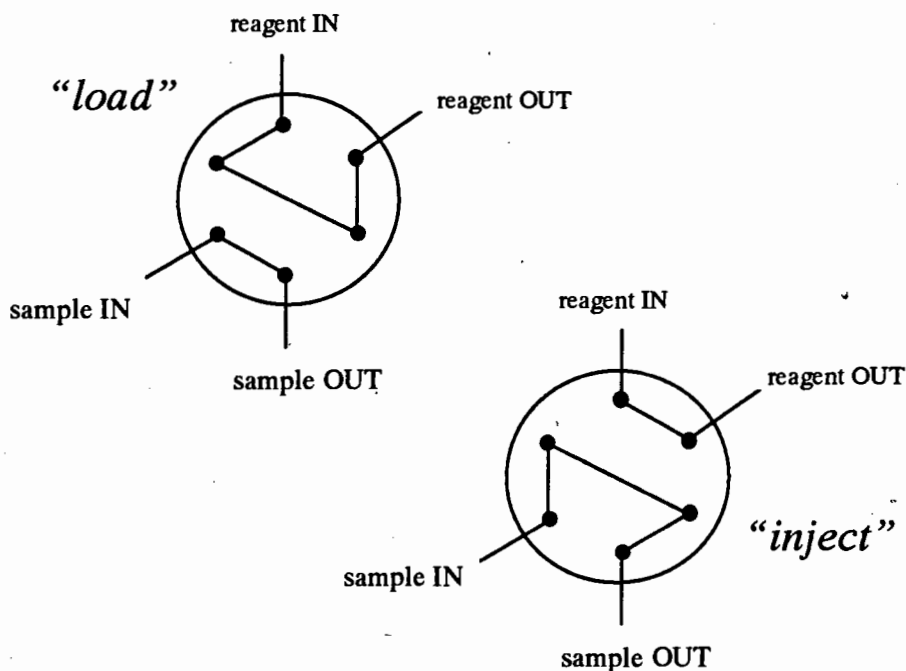


Figure 4.7 Schematic of a 6-port injection valve showing the reagent “load” and “inject” positions for a reverse-FIA manifold. The sample forms the carrier stream.

The effect of the reagent injection volume on the sensitivity and calibration slope is recorded in Table 4.2.

The sensitivity of the system increased with increasing sample injection volumes. Coloured zones were observed in the injected reagent zone when larger reagent injection volumes were used. This is a result of insufficient mixing and reaction of the sample and reagent components in the centre of this zone. Poor reproducibility was found for these larger volumes. Calibration slopes were lower when using large injection volumes.

Table 4.2 *The effect of the reagent injection volume on (1) the response obtained for a $40 \mu\text{g.cm}^{-3}$ ascorbic acid standard, (2) the calibration slope, and (3) the correlation coefficient of the calibration.*

| Injection Volume (μl) | Absorbance (AU) | Slope (x 1000) (AU/ $\mu\text{g.cm}^{-3}$) | Corr. Coeff. (r) |
|---------------------------------------|--------------------|--|------------------------|
| 229 | 0.707 | -1.27 | 0.9983 |
| 179 | 0.480 | -1.95 | 1.0000 |
| 78 | 0.257 | -2.66 | 0.9990 |

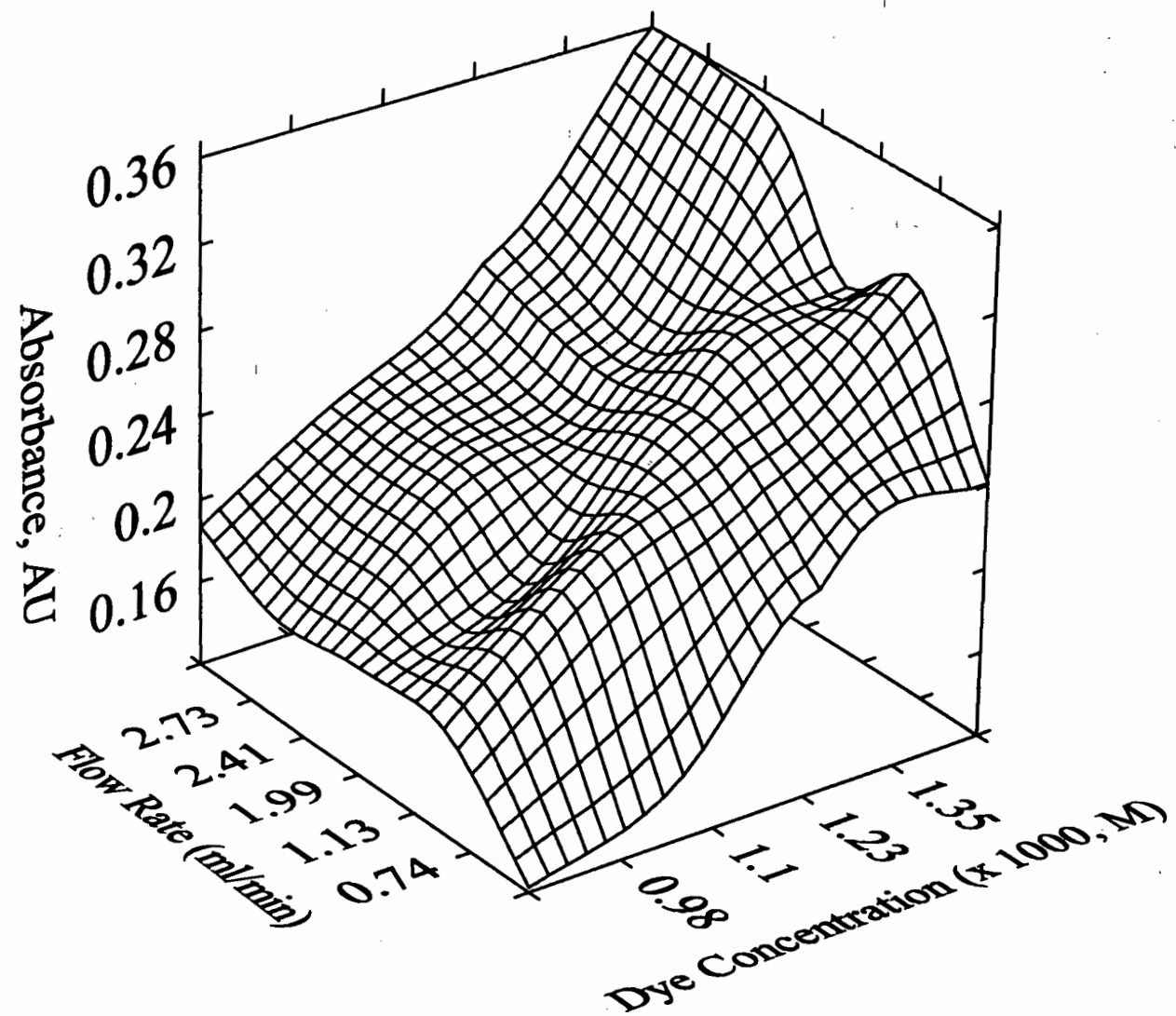
The smallest sample injection volume (78 μl) showed adequate sensitivity and excellent reproducibility (RSD = 0.85 %, n = 5). A narrower peak width than those observed in the larger injection volumes was observed. Less peak tailing and carry-over and a higher sampling rate resulted. The calibration slope for this injection volume was the highest. Consequently, 78 μl was selected as the reagent injection volume.

4.3.1.5 Reagent Concentration

Dye solutions of the following concentrations were used to optimise the reagent concentration : (a) $0.98 \times 10^{-3} \text{ M}$, (b) $1.10 \times 10^{-3} \text{ M}$, (c) $1.23 \times 10^{-3} \text{ M}$ and (d) $1.35 \times 10^{-3} \text{ M}$ 2,6 DCPIP. A $40 \mu\text{g.cm}^{-3}$ ascorbic acid standard was used in this optimisation.

The dye concentrations were injected into the ascorbic acid carrier stream using different pump flow rates. A three dimensional surface plot (Figure 4.8) shows the effect of flow rate and dye concentration on the response of the system.

Figure 4.8 *Three dimensional surface plot showing the effects of 2,6 DCPIP concentration and flow rate on the response of a $40 \mu\text{g}.\text{cm}^{-3}$ ascorbic acid solution.*



The 3-dimensional surface of the plot clearly indicates an increase in sensitivity as the concentration of the dye increases. The flow rates show a slight increase in sensitivity at each dye concentration examined. This is accounted for by the extent of zone penetration and therefore mixing between the reagent and sample solutions.

High concentrations of 2,6 DCPIP resulted in the formation of solid particulate matter that deposited within the reaction coil. The higher the dye concentration, the faster the particulate matter formed. The solid matter caused blockages in the system. In order to minimise reagent cost and to reduce the problems caused by formation of solid matter in the reaction coil, the system was operated using a lower dye concentration of $1.23 \times 10^{-3} M$, despite a slight decrease of sensitivity.

4.3.2 Final Manifold for the 2,6 DCPIP-Ascorbic Acid Method

Final parameters used in the single line manifold (Figure 4.9) for the flow-injection system are listed : C - ascorbic acid carrier streams (varying concentrations); F - flow rate ($1.99 \text{ cm}^3 \cdot \text{min}^{-1}$); S - sample injection (78 μl); I - dye injection ($1.23 \times 10^{-3} M$); RC - reaction coil length (100 cm, 0.5 mm i.d.); D - spectrophotometer (518 nm); W - waste. All experiments were carried out at room temperature. (Detail of the apparatus is tabulated in Appendix E.)

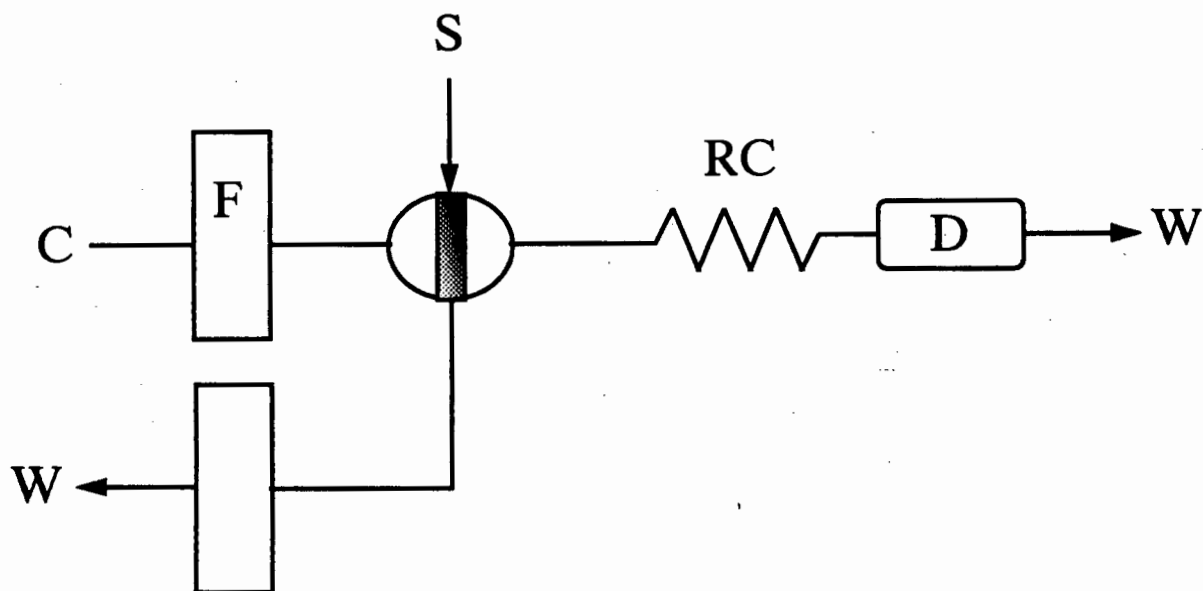


Figure 4.9 Final manifold and parameters for the determination of ascorbic acid by FIA using 2,6 DCPIP. See text for details.

Once the optimum parameters had been established for the 2,6 DCPIP-ascorbic acid FIA, the optimal conditions were tested and the results detailed in the following section.

4.3.3 Examination of Optimal Conditions

Optimum conditions were tested with regard to (a) repeatability (i.e. “within sample” precision), (b) reproducibility (i.e. “between sample” precision) and (c) carry-over.

4.3.3.1 Repeatability

Within sample precision for the experimental procedure was determined as follows. A $20 \mu\text{g}\cdot\text{cm}^{-3}$ ascorbic acid standard was injected into the system 10 times in order to obtain the RSD. The RSD was 0.71 %. The manifold configuration thus yields highly repeatable results.

Standards of higher concentrations ($60 \mu\text{g.cm}^{-3}$ and $100 \mu\text{g.cm}^{-3}$) were also injected in the same manner in order to confirm the repeatability of the experiment. RSD values were found to be acceptable in both cases, confirming the repeatability of the manifold.

4.3.3.2 Reproducibility

A $20 \mu\text{g.cm}^{-3}$ ascorbic acid standard was injected 10 times over a period of 5 days. The daily variance in absorbance was found to be low considering that fresh solutions for each analysis had to be prepared (Table 4.3).

Table 4.3 *Daily variation in absorbance.*

| Day | Absorbance (AU) |
|-------|-----------------|
| Day 1 | 0.255 |
| Day 2 | 0.249 |
| Day 3 | 0.250 |
| Day 4 | 0.249 |
| Day 5 | 0.253 |

4.3.3.3 Carry-Over and Sampling Rate

Alternate injection of the reagent dye into low ($20 \mu\text{g.cm}^{-3}$) and high ($100 \mu\text{g.cm}^{-3}$) concentration carrier streams showed that there was no sample carry-over between experiments. The sampling rate for the developed method was 40 samples per hour. The method may also be readily automated by using computer control of the injection valve and adding a selection valve to select either ascorbic acid standard solutions or urine samples.

4.4 Experimental Procedure and Demonstrative Results

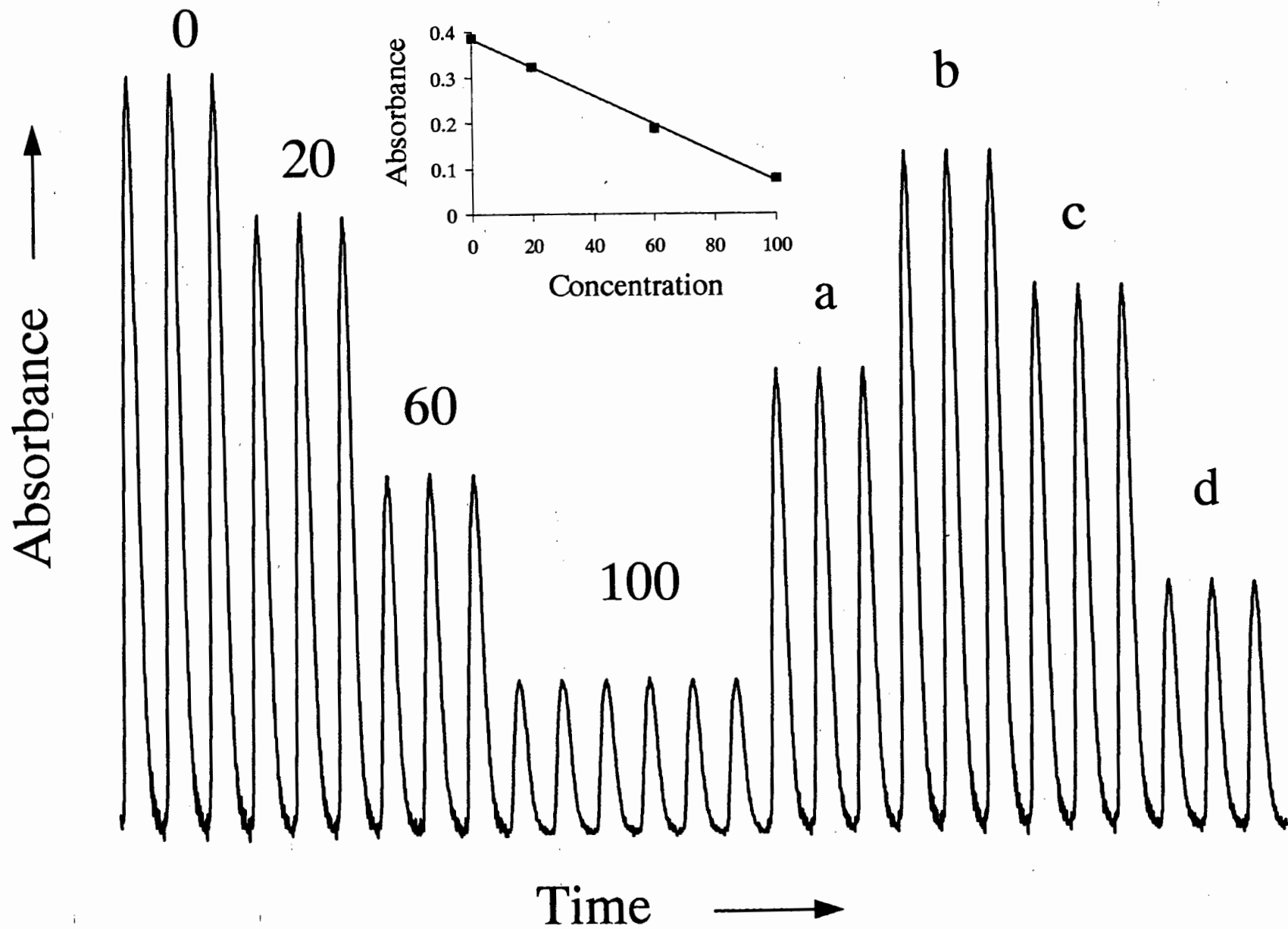
Ascorbic acid determination by FIA was carried out in conjunction with the manual titration method (Chapter 3). The optimised manifold established for the measurement of ascorbic acid excreted in urine by FIA enabled measurement of ascorbic acid concentration to a maximum of $150 \mu\text{g}\cdot\text{cm}^{-3}$.

4.4.1 *Experimental Procedure*

Individuals participating in the ascorbic acid experiments collected urine directly into glacial acetic acid at specific urine voidings (voiding "x", voiding "y" and voiding "z") during the 24 hour collection period (Section 3.2). Ascorbic acid excreted in the urine was initially determined in each of the aliquots by means of a manual titration. In instances where the ascorbic acid concentration exceeded the range of measurement allowed by FIA, the urine was suitably diluted using a prepared solution of acetic acid. The urine sample was manually titrated to ensure that the ascorbic acid concentration did indeed fall below the maximum concentration tolerable by the FIA method.

The analogue output port of the detector was coupled to a computer data acquisition package (FlowTEK™, Mintek, Randburg, South Africa) to collect and display the peak profiles as shown in Figure 4.10. The calibration curve for a range of standards is shown as an inset. Some urine sample solutions were also analysed and are shown.

Figure 4.10 *Typical replicate profiles using the optimised manifold. The numerals refer to ascorbic acid concentrations in $\mu\text{g.cm}^{-3}$. Letters correspond to (a) 43.6, (b) 9.6, (c) 30.7, and (d) 81.1 $\mu\text{g.cm}^{-3}$ ascorbic acid in urine samples. The calibration curve is shown as an inset.*



4.4.2 Demonstrative Results

The ascorbic acid concentrations determined from a calibration plot, for all the aliquots collected by 2 of the 10 participants during the twelve day experimental period are given in Table 4.4. The ascorbic acid concentrations determined in the same urine aliquots using the 2,6 DCPIP manual titration method are also shown. (A full set of results for these analyses are given in Chapter 6).

Table 4.4 *Urinary ascorbic acid excretion values determined in the urine of 2 of the 10 participants during the full twelve day experimental period measured by : (a) manual titration (MT) and (b) flow-injection analysis (FIA).*

| | RG | | AM | | | RG | | AM | |
|----------|-------|-------|-------|-------|----------|-------|-------|------|------|
| | MT | FIA | MT | FIA | | MT | FIA | MT | FIA |
| #1 v_x | - | 62.8 | 112.1 | 137.0 | #5 v_x | 75.5 | 67.6 | 68.8 | 54.3 |
| v_y | - | 5.7 | 72.8 | 78.9 | v_y | 161.6 | 136.1 | 58.3 | 47.3 |
| v_z | - | 24.2 | 86.8 | 178.8 | v_z | 87.3 | 77.4 | 45.4 | 45.7 |
| #2 v_x | 27.6 | 57.3 | 113.4 | 155.5 | #6 v_x | 49.0 | 49.5 | 34.7 | 45.3 |
| v_y | 19.9 | 43.5 | 106.7 | 142.4 | v_y | 72.0 | 76.1 | 69.8 | 76.1 |
| v_z | 10.0 | 23.4 | 43.5 | 79.9 | v_z | 96.7 | 91.7 | 10.0 | 7.8 |
| #3 v_x | 82.1 | 122.9 | 52.6 | 47.1 | #7 v_x | 10.0 | 29.3 | 10.0 | 11.0 |
| v_y | 53.4 | 89.4 | 84.6 | 132.9 | v_y | 45.3 | 62.9 | 10.0 | 16.6 |
| v_z | 85.5 | 146.5 | 64.6 | 108.3 | v_z | 25.2 | 50.8 | 10.0 | 19.7 |
| #4 v_x | 119.0 | 93.7 | 89.2 | 78.2 | #8 v_x | 29.1 | 60.8 | 10.0 | 20.5 |
| v_y | 93.1 | 84.8 | 86.9 | 80.4 | v_y | 45.2 | 86.2 | 10.0 | 17.7 |
| v_z | 69.25 | 58.8 | 48.6 | 46.8 | v_z | 10.0 | 19.0 | 10.0 | 9.41 |

1 - 8 : sample number (Table 6.1)

v_x , v_y , v_z : voiding times x, y, and z (Table 6.19)

RG, AM : participants' initials

4.4.3 *Statistical Evaluation and Analysis of Results*

There are two aspects to be considered in the comparison of the methods :

- (a) How repeatable are the measurements for each method ?
- (b) Are the methods comparable, in other words, do they give the same results ?

4.4.3.1 Repeatability

For each method the mean ($n = 3$) ascorbic acid concentration of the sample was plotted against the standard deviation of the sample. The mean and the standard deviation were found to be independent for each method. A one-way analysis of variance (ANOVA) was carried out to obtain a measure of the repeatability. The British Standards Institution defines a coefficient of repeatability as being twice the standard deviation [10]. The manual titration method had a coefficient of 71.0 and FIA had a coefficient of 57.4. The F-test of the variances for each method showed that FIA had a significantly smaller variance ($F = 1.53$, $P < 0.05$) than does the manual titration method.

It is concluded from this analysis that the repeatability of the flow-injection method is superior to that of the manual titration method (i.e. repeated measurements are less variable). This is in accordance with the expectations. FIA is inherently highly reproducible due to the reproducible nature of the sample handling procedure within the manifold tubing. Furthermore, the use of a spectrophotometer to monitor the reaction “pseudo-end point” removes any bias arising from the monitoring of a colour change during titration by eye.

4.4.3.2 Comparison of the Methods

It is desirable to establish whether the methods are comparable with a view to the replacement of the more tedious titration by the simple FIA approach.

The statistical approach was to plot the differences of the sample means for each method as a function of the average of the two methods (Appendix F). The values close to zero were taken to be zero for the purposes of this plot. Confidence limits at 95 % have been drawn on

the plot. Four of the seventy-six points fall out of these limits, which is acceptable since statistically five out of a hundred points are expected to do so [10]. The plot enables the assessment of the magnitude of any possible disagreement between the methods in terms of bias and error.

There is a significant bias : Ascorbic acid concentrations determined by FIA give concentrations that are of the order of 6.5 % higher than those concentrations determined by manual titration (t -value = 3; n = 76; p = 0.0021). This probably arises as a result of errors in the visual determination of the end point using the manual titration method. As discussed previously, the end point is extremely difficult to detect by eye (Section 2.5.1). To ensure that all the colour had been discharged from the reaction vessel there was a tendency to over-titrate as the endpoint was approached. This would result in lower ascorbic acid concentrations being found than when analysed by FIA.

There is no evidence of a relationship between the differences and the averages (correlation, r = -0.1498, n = 76, p = 0.1964) of the two methods, indicating that the accuracy of the methods is similar. If this correlation were significant, it would indicate that there was a difference in the total variance of the measurements obtained by the two methods. The sample means for the manual titration method have standard deviations of 39.4 (if the values below $10 \mu\text{g}\cdot\text{cm}^{-3}$ are included in the mean) and 37.5 (if these low ascorbic acid concentrations are excluded from the mean) while the standard deviation for the flow-injection technique is 36.8.

Further validation of the accuracy may be done by using a third method for the determination of ascorbic acid involving an enzyme assay (Boehringer Mannheim, Cat. No. 409 677).

4.5 Conclusions

The design and development of a new approach to an existing titrimetric method for the determination of ascorbic acid in urine by 2,6 DCPIP has been successfully demonstrated. The flow-injection based method gives results statistically similar to the existing manual batch titration. The developed FIA method is more economical, rapid and is easy to implement.

Although the methods are similar in accuracy, FIA yields results which are more repeatable than those obtained by the manual titration method. The results obtained with the manual titration indicate over-titration of the urine which results in lower ascorbic acid concentrations being found. Spectrophotometric detection is used in the flow-injection method, and therefore the results obtained are more precise than those obtained by manual titration since bias associated with the end point detection is removed.

The specificity of 2,6 DCPIP for ascorbic acid in the manual titration is known to be a limiting factor (Section 2.5.1). However, when using FIA and a "pseudo-end point", this limitation is significantly reduced due to the shorter reaction time allowed for any such interference to occur prior to analyte detection. Additional dilution in FIA of these interferents also increases the specificity.

Further work is required to validate the accuracy of the method by comparison against another technique (e.g. enzyme assay). The use of on-line standard additions would serve to enhance the validity of the results in the complex urine matrix. It is envisaged that a dedicated FIA analyser under computer control, with an autosampler, could be developed and this would allow continuous measurement of ascorbic acid in a series of samples. No doubt such an analyser, and others like it, will become powerful tools in biochemical and biomedical laboratories.

4.6 References

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Chapter 5.

Haematuria after Megadose Ingestion of Ascorbic Acid - A Case Study

5.1 Introduction

A 25 year old, white male with no history of stone formation was the first participant in the investigation to determine the effects of ascorbic acid on the risk of kidney stone formation. The participant was required to ingest 8 g of ascorbic acid daily [1], whilst following his normal daily diet, in a protocol which was planned to last 9 days. The 8 g load of ascorbic acid was divided into 2 g doses taken four times daily : at breakfast, lunch, dinner and just before retiring.

The participant collected a 24 hour urine prior to commencing the protocol to serve as a control. Ingestion of ascorbic acid then commenced and was continued for a further 8 days. Twenty-four hour urines were collected on the 1st and 5th days of the protocol to establish the short and long term effects of ascorbic acid on urinary oxalate and calcium oxalate crystallization.

5.2 Detection of Haematuria

Significant haematuria (blood spotting, later confirmed with a Boehringer Mannheim dip stick) was detected on the 8th day of the protocol. As a result, further ingestion of ascorbic acid was immediately suspended. Fluid intake was increased in order to dilute the urine as much as possible. Twenty-four hour urines were collected during the 24 hour period immediately following the detection of haematuria and again 5 days after the protocol was halted (i.e. during day 1 and day 6 post cessation of ascorbic acid ingestion).

The 24 hour urine sample collected immediately after cessation of the ascorbic acid protocol was tested for the presence of blood using Boehringer Mannheim dip sticks. Aliquots were dispatched for full pathological analysis. A small volume of the sample was centrifuged and prepared for scanning electron microscopy (SEM). The final voiding of the 24 hour period was collected separately and analysed for blood, before being added to the 24 hour volume.

5.3 Protocol Re-Evaluation

The detection of haematuria in the urine of the first participant indicated the very real dangers of a high ingestion of ascorbic acid for an extended period. Although the literature shows conflicting opinions regarding the effects of ascorbic acid in calcium oxalate urolithiasis [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11], the potential danger of the vitamin could have been accentuated by the protocol used.

As a result, the original protocol selected for the present study was altered to the ingestion of only 4 g of ascorbic acid for a period of 5 days (Section 3.1.2).

5.4 Results and Discussion

The individual in the present study collected 24 hour samples as described previously. In order to simplify the presentation of the results and discussion, each collection has been designated a sample number. Table 5.1 identifies the day on which each collection was effected with the corresponding sample number.

Table 5.1 *Definition of sample collections.*

Sample 1 = control collection

Sample 2 = first day ascorbic acid ingestion, 24 hour urine collection

Sample 3 = fifth day ascorbic acid ingestion, 24 hour urine collection

Sample 4 = first day post cessation of ascorbic acid ingestion, 24 hour urine collection

Sample 5 = sixth day post cessation of ascorbic acid ingestion, 24 hour urine collection

5.4.1 Urinary Components

The volume, pH and chemical composition of each urine were determined for the participant prior to, during and after the ascorbic acid protocol. These data are shown in Table 5.2.

Table 5.2 *Urinary excretions (mmol/24 hr), pH and volume (cm³/24 hr) during the 15 day experimental period.*

| # | Mg | Ca | Na | K | Vol | pH | Phos | Creat | Cit | Ox | Urate | Cl |
|---|------|------|-----|------|------|------|------|-------|------|------|-------|-----|
| 1 | 3.78 | 2.13 | 163 | 61.6 | 1760 | 6.55 | 30.8 | 12.7 | 2.06 | 0.14 | 2.43 | 190 |
| 2 | 5.76 | 4.50 | 129 | 41.9 | 1340 | 5.55 | 30.0 | 14.7 | 2.15 | 0.14 | - | 127 |
| 3 | 6.15 | 3.25 | 76 | 35.0 | 1675 | 5.55 | 22.4 | 13.5 | 2.05 | 0.34 | - | 51 |
| 4 | 3.23 | 3.21 | 87 | 61.8 | 1680 | 5.85 | 23.5 | 13.8 | 2.14 | 0.20 | 1.41 | 111 |
| 5 | 3.15 | 2.00 | 128 | 56.9 | 1440 | 6.35 | 25.5 | 14.5 | 3.14 | 0.21 | 3.19 | 112 |

An immediate increase in urinary magnesium excretion occurs after a 24 hour period of ascorbic acid ingestion (sample 2). After 5 days of ascorbic acid ingestion, a further increase in magnesium excretion is observed (sample 3) but values return to that of the control after cessation of the protocol (samples 4 and 5). Since magnesium is a known inhibitor [12, 13, 14], the ingestion of ascorbic acid causes a decrease in the stone forming potential of the urine with respect to this risk factor.

A noticeable increase in urinary calcium excretion occurs after the initial ingestion of 8 g of ascorbic acid (sample 2). After a prolonged period of ascorbic acid ingestion, urinary calcium levels are seen to decrease (sample 3) and continue to do so after the cessation of ascorbic acid ingestion (samples 4 and 5). After 5 days in which no ascorbic acid was ingested, calcium excretion in the urine was similar to the levels observed for the control collection (sample 1). Thus, megadose ingestion of ascorbic acid initially increases the stone forming risk of the urine, but the effect is not maintained.

The initial ingestion of ascorbic acid causes a decrease urinary sodium, potassium and chloride excretion (sample 2). Urinary sodium, potassium and chloride levels decrease further after a prolonged period of ascorbic acid ingestion (sample 3). The excretion of these urinary components increased slightly after the interruption of the protocol (sample 4). While potassium levels remain consistently low, sodium and chloride excretion increases 5 days after the cessation of ascorbic acid ingestion (sample 5), but they do not return to control (sample 1) values, suggesting that megadose ingestion of the vitamin has carry-over effects on the excretion of these three components. This is particularly significant in the case of potassium which has been shown to cause an increase in the urinary excretion of calcium, resulting in an increased risk of kidney stone formation [15]. This is consistent with the findings in sample 2, as mentioned previously.

The decrease in urinary pH during the ingestion period of ascorbic acid is expected due to the acidic nature of the vitamin. However, after the cessation of the protocol, the pH returns to that observed in the control sample. Thus, ascorbic acid ingestion does not have any long term effects on the pH of the urine. No particular trend is observed for urinary volume.

Previous work has shown that stone formers exhibit lower excretion values of ascorbic acid and citrate [16] and that citrate hinders the uptake of ascorbic acid due to competition by the two components for the same carrier in the gut [16]. A number of other studies have shown that citrate inhibits calcium oxalate growth [17] and aggregation [18, 19, 20]. Table 5.2 shows that citrate excretion is unaffected by the ingestion of large doses of ascorbic acid. Thus, inhibitory activity and the stone forming potential of the urine are unaffected.

Urinary creatinine excretion shows no trend during the ascorbic acid protocol nor during the period thereafter. Creatinine levels are thus unaffected by megadose ingestion of ascorbic acid.

As discussed in Section 3.3, urate levels could not be determined with confidence in conditions of raised ascorbic acid levels due to the masking effect of the vitamin in the analysis.

Urinary phosphate levels decreased during the ingestion period but started to recover after the cessation of the protocol. Hence, ingestion of megadose quantities of ascorbic acid resulted in a decrease in urinary phosphate. Previous studies have shown that phosphate depletion can increase urinary calcium [21, 22]. In the present study, urinary calcium increased significantly with initial ingestion of ascorbic acid but did not remain consistently raised. Phosphate depletion can thus be associated with an initial increase in calcium levels and hence an initial increase in the stone forming potential of this individual's urine. The continued depletion of urinary phosphate during the protocol is noteworthy since reduced levels have been associated with an increased stone risk.

5.4.2 *The Metastable Limit*

The calcium oxalate metastable limits determined by turbidimetry and Coulter multisizer methods for each urine are given in Table 5.3. It can be seen that a decrease occurs after the initial ingestion of 8 g ascorbic acid. Continued megadose ascorbic acid ingestion for a period of 5 days caused a slight increase in the metastable limit but the value is still much lower than that for the control urine. Six days post cessation of ascorbic acid ingestion, the metastable limit of the urine had returned to its control value. Table 5.2 shows that the trend for the

metastable limit, irrespective of the method employed, was the same. This indicates the compatibility of the two techniques.

Thus megadose ascorbic acid ingestion caused a lowering of the calcium oxalate metastable limit in this individual. Hence, during the protocol the nucleating potential of the urine would have been increased.

Table 5.3 *Metastable limits determined by turbidimetry and Coulter multisizer.*

| | Turbidity (mol.dm ⁻³) | Coulter Multisizer (mol.dm ⁻³) |
|----------|-----------------------------------|---|
| Sample 1 | 0.14 | 0.11 |
| Sample 2 | 0.05 | 0.05 |
| Sample 3 | 0.07 | 0.07 |
| Sample 4 | 0.08 | 0.08 |
| Sample 5 | 0.14 | 0.12 |

5.4.3 Urinary Oxalate Excretion

Urinary oxalate was determined in the bulk 24 hour sample and in the representative sample containing 10 mmol.dm⁻³ EDTA preservative (Table 5.4).

Urinary oxalate excretion increased during the 5 day period of ascorbic acid ingestion relative to its pre-protocol value. The sudden interruption of the protocol prevented the oxalate excretion in the urine being determined after 9 consecutive days of ascorbic acid ingestion. The urinary oxalate excretion decreased during the 24 hour period post cessation ascorbic acid ingestion, but a further decrease was not observed 6 days later. Thus urinary

oxalate remained slightly raised with respect to the control excretion values. The trend suggests that ingestion of ascorbic acid results in *in vivo* conversion of ascorbic acid to oxalic acid, thereby increasing the stone forming potential in the urine of this individual.

Table 5.4 *Urinary oxalate excretion (mmol/24 hours) during the 15 day experimental period.*

| | Bulk 24 hr Sample | Representative Sample + EDTA |
|----------|-------------------|---------------------------------|
| Sample 1 | 0.141 | 0.114 |
| Sample 2 | 0.140 | 0.136 |
| Sample 3 | 0.340 | 0.410 |
| Sample 4 | 0.200 | 0.210 |
| Sample 5 | 0.210 | 0.200 |

It is noted that the representative samples with EDTA are not significantly different to unpreserved samples, indicating that this participant's urine did not undergo *in vitro* conversion of ascorbic acid to oxalic acid. However generalisations cannot be made. While conversion of ascorbate to oxalate might occur in some urine, its complex nature plays an important role in determining the extent to which *in vitro* conversion occurs. Thus, the conversion occurs to a lesser extent in some urines while in others it does not occur at all.

5.4.4 Ascorbic Acid Concentration

Ascorbic acid concentrations were measured using the 2,6 DCPIP manual titration. FIA could not be used to determine ascorbic acid concentrations in urines with high ascorbic acid

loads since FIA determination of the vitamin concentrations was limited to a maximum concentration of $150 \mu\text{g}\cdot\text{cm}^{-3}$. Urines were diluted after the total ascorbic acid concentration had been determined in order to compare the results obtained by the two methods.

A noticeable increase in ascorbic acid concentration occurs after the ingestion of 8 g of ascorbic acid (Table 5.5). After 5 days of continued ingestion, the concentration of urinary ascorbic acid has not increased further and might have reached a plateau value. It has been previously recorded that reabsorption of ascorbic acid by the gut is limited to $2.16 \text{ cm}^3\cdot\text{min}^{-1}$ [23] and that when the vitamin is presented to the kidney tubules at a rate greater than this, the excess ascorbic acid is excreted in the urine. As the dose increases, so the absorption decreases and the process eventually attains a level of supersaturation [24]. Unfortunately the sudden interruption of ascorbic acid ingestion prevented a third measurement of ascorbic acid concentration. This would have permitted investigation of possible plateau formation in the concentration level. However, since an immediate increase in ascorbic acid concentration occurred after the initial ingestion of 8 g ascorbic acid and appeared to level off as the protocol proceeded, it is suggested that this individual probably absorbed only that amount of ascorbic acid which his body required while the remainder was excreted. A level of supersaturation was thus probably attained with excess ascorbic acid being excreted.

Table 5.5 shows the mean ascorbic acid concentration (voiding "x", "y" and "z") for the participant collected as discussed in Section 3.2. Ascorbic acid concentration in the urine decreased considerably in the 24 hour period immediately post ascorbic acid ingestion and that a further decrease is observed 6 days after the ascorbic acid ingestion was suspended. These decreases in concentration are probably due to suspension of ascorbic acid ingestion as well as to increased urinary volumes as the participant was instructed to increase his fluid intake immediately after haematuria had been detected.

Table 5.5 *Mean ascorbic acid concentrations for three specified voidings (over 24 hours) determined by manual titration at various stages of the 12 day experimental period.*

| Ascorbic Acid Concentration ($\mu\text{g.cm}^{-3}$) | |
|--|------|
| Sample 1 | 152 |
| Sample 2 | 1852 |
| Sample 3 | 1654 |
| Sample 4 | 242 |
| Sample 5 | 38 |

The adult human body accumulates a limited amount of ascorbic acid (normally 2 - 3 g) which can rise to a maximum of 4 g [25]. At this point, any intake in excess of the daily rate of metabolism is absorbed but promptly excreted unchanged through the kidneys [25]. That which is metabolised is broken down in the liver, and to some extent also by the kidney, in a series of reactions culminating in oxalic acid, which is excreted in the urine [25]. From a dietary point of view, ascorbic acid intake is viewed primarily as being required to prevent scurvy with as little as 10 mg daily being effective in this respect. The recommended daily allowance (RDA) varies : 30 mg in the United Kingdom [25], 60 mg according to Gowenlock *et al.* [26], and according to laboratory rat ascorbic acid tests, an amount has been calculated that is equivalent to 2 g [25]. The daily output of ascorbic acid is approximately half the minimum intake, usually about 20 - 30 mg [26]. After 5 days post cessation ascorbic acid ingestion urinary ascorbic acid levels had almost returned to the expected daily output levels of the vitamin.

There are a number of problems associated with experimental analysis of urinary ascorbic acid. Firstly, analysis must be performed on *fresh* samples (even if a stabilising preservative such as EDTA or glacial acetic acid is present) [26]. Thus, the total amount of ascorbic acid

excreted in the urine in a 24 hour period cannot be determined easily. Secondly, the presence of the preservative which is necessary to prevent the conversion to oxalate prevents certain other investigations (e.g. crystallization experiments) being performed since chelation of urinary calcium by EDTA occurs. The concentrations were averaged for the three ascorbic acid samples in each 24 hour collection since this appeared to be the most practical way to report the data.

5.4.5 Tiselius Risk Index and CaOx Relative Supersaturation

The Tiselius risk index [27] was determined for each 24 hour urine collection. The results are presented in Table 5.6. It is noted that the risk index increased after the initial ingestion of 8 g ascorbic acid and that a further increase occurred after the participant had ingested 8 g ascorbic acid daily for a period of 5 days. During the 24 hour period immediately post cessation of ascorbic acid ingestion the value decreased and a further decrease was observed 6 days later. The trend of the Tiselius biochemical risk index observed in this individual indicates that prolonged ascorbic acid ingestion caused an increase in the stone forming potential of the urine.

Table 5.6 *Tiselius risk index and calcium oxalate relative supersaturation values.*

| | Tiselius Risk Index | CaOx Relative Supersaturation |
|----------|------------------------|----------------------------------|
| Sample 1 | 114.2 | 1.097 |
| Sample 2 | 146.0 | 3.150 |
| Sample 3 | 317.5 | 5.456 |
| Sample 4 | 195.3 | 3.761 |
| Sample 5 | 131.6 | 1.975 |

The calcium oxalate relative supersaturation values [28] determined for each 24 hour sample show the same trend as that observed for the Tiselius risk index (Table 5.6). This provides further evidence supporting the observations that prolonged ingestion of megadose quantities of ascorbic acid increased the risk of stone formation in this individual.

5.4.6 *Scanning Electron Microscopy*

Scanning electron microscopy (SEM) observations have been summarised in Table 5.7. Very few calcium oxalate dihydrate (COD) single crystals or aggregates were observed prior to the development of haematuria (samples 1, 2 and 3, Table 5.7). However, varying amounts of epithelial debris and organic mucoid material were detected in these samples. In the single sample voiding collected 12 hours after the detection of haematuria, numerous single COD crystals (~5 μm cross section) were noted, together with large, jagged COD crystal aggregates (25 μm to 100 μm cross section). Typical examples of individual and aggregated COD crystals are shown in Figures 5.1, 5.2 and 5.3. The sample collected 24 hours after cessation of the protocol showed the presence of a few isolated single COD crystals as well as a few isolated crystal aggregates. These were much smaller than those observed in the sample collected 12 hours after the detection of haematuria and displayed much "cleaner" crystal surfaces due to the relative absence of organic mucoid material (Figure 5.4). The sample analysed 6 days post cessation of ascorbic acid ingestion showed no crystalline or organic mucoid material.

Table 5.7 *A summary of the observations by SEM analysis.*

| | Single COD's | Aggregate COD's | Epithelial Debris | Organic Matter | Blood Cells |
|--------------------|------------------------|-------------------------------|----------------------|-------------------|----------------|
| Sample 1 | α | α | β | β | α |
| Sample 2 | α | α | β | β | α |
| Sample 3 | α | α | β | β | α |
| 12 hrs Post Cessn. | δ (~ 5 μ m) | δ (~ 25 - 100 μ m) | β | β | β |
| Sample 4 | β | β | α | α | α |
| Sample 5 | α | α | α | α | α |

α = not detected

β = small quantities detected

δ (~ X μ m) = substantial visible particulate matter

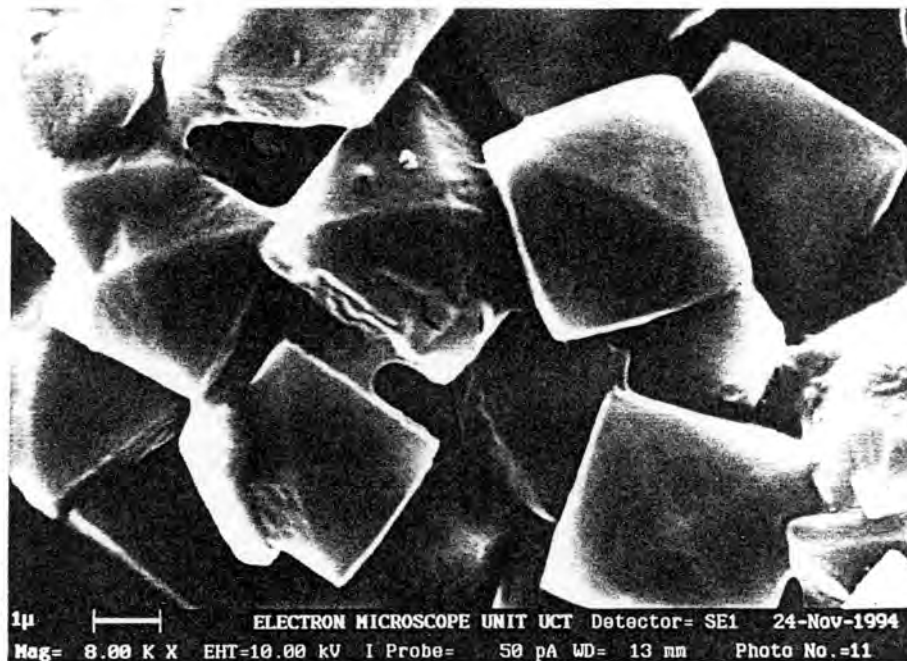


Figure 5.1 *Single COD crystals (~5 μ m in cross section) covered in an organic mucoid layer observed in urine collected 12 hours after the detection of haematuria.*

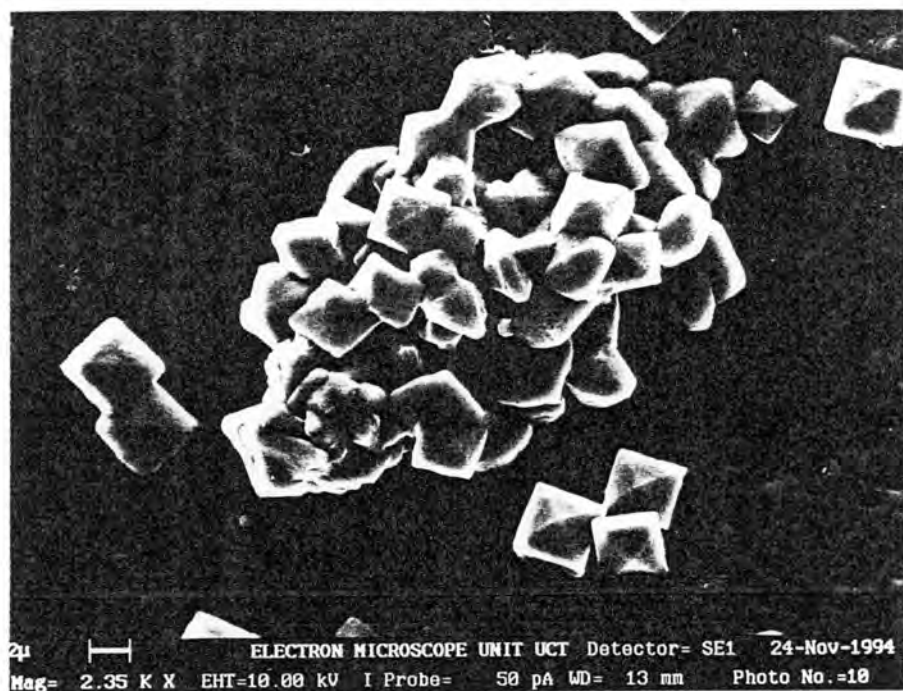


Figure 5.2 *COD crystal aggregate (~25 μm in cross section) observed in urine collected 12 hours after the detection of haematuria.*

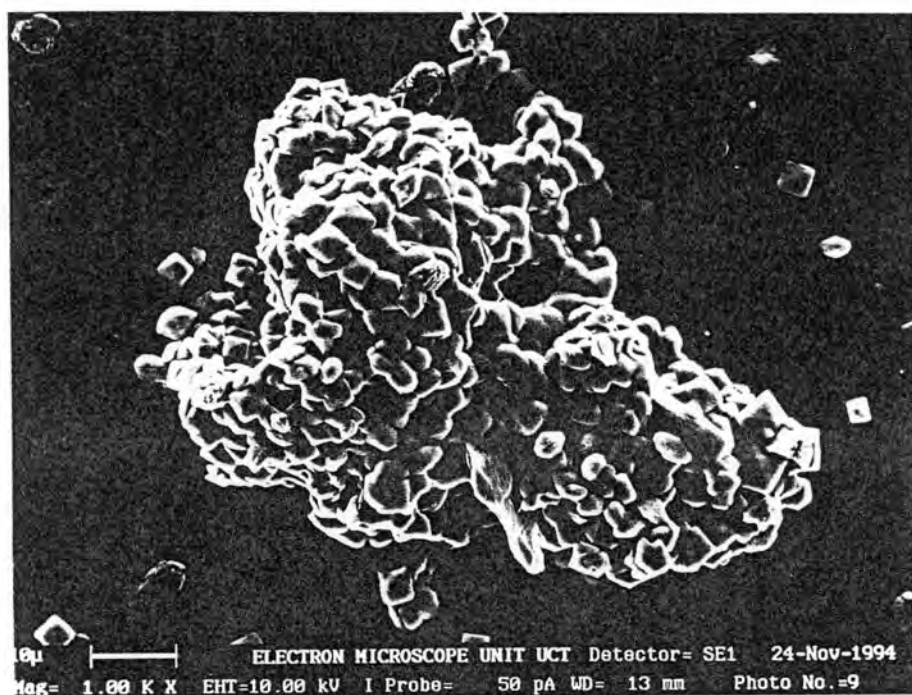


Figure 5.3 *Gigantic COD crystal aggregates (~100 μm in cross section) observed in urine collected 12 hours after the detection of haematuria.*

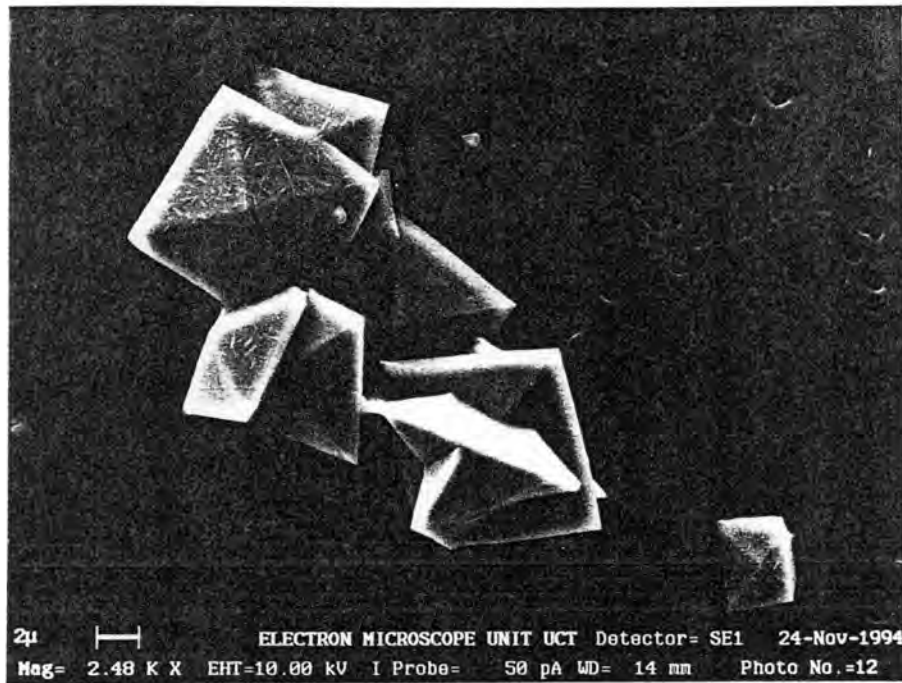


Figure 5.4 *Small, isolated COD crystal aggregates (~25 μm in cross section) observed in urine collected 24 hours after cessation of the protocol.*

5.5 Conclusions

The results of this case study demonstrate that ascorbic acid ingestion induced calcium oxalate crystalluria in this individual. The passage of the crystals and aggregates thereof probably caused irritation and epithelial injury manifesting as haematuria. Although this individual's response to ascorbic acid ingestion is probably rare, it should be brought to the attention of stone researchers as high dosage of ascorbic acid by such individuals could have undesirable results.

Within 8 months of these investigations, the individual concerned notified the Kidney Stone Research Unit (U.C.T) on further developments related to his health. As a result of severe, pulsing lower back pain he consulted a general practitioner. Pathological investigation indicated haematuria in the urine. Ultra-sound scans showed significant inflammation of the ureter just below the kidney suggesting that the individual had probably passed gravel or a small kidney stone(s). The health of the individual will continue to be monitored.

5.6 References

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Chapter 6.

Experimental Results

The 10 participants collected 24 hour samples as described previously. In order to simplify the presentation of the results and discussion, each collection has been designated a sample number. Table 6.1 describes the collection procedure corresponding to each sample number.

Table 6.1 *Description of sample collection procedures followed.*

Sample 1 = control collection

Sample 2 = control collection

Sample 3 = first day ascorbic acid ingestion, 24 hour urine collection

Sample 4 = third day ascorbic acid ingestion, 24 hour urine collection

Sample 5 = fifth day ascorbic acid ingestion, 24 hour urine collection

Sample 6 = first day post cessation ascorbic acid, 24 hour urine collection

Sample 7 = third day post cessation ascorbic acid, 24 hour urine collection

Sample 8 = fifth day post cessation ascorbic acid, 24 hour urine collection

In the statistical analysis, the samples were grouped into the following categories :

- samples 1 and 2 : samples collected prior to ascorbic acid ingestion
- samples 3, 4 and 5 : samples collected during the period of ascorbic acid ingestion
- samples 6, 7 and 8 : samples collected in the period post cessation ascorbic acid ingestion

6.1 Urinary Components

The volume, pH and chemical composition of each urine were determined for each participant prior to, during and after the ascorbic acid experimental period. The total excretion (mmol per 24 hours) of each component for respective participants are shown in Appendix D. Mean excretions, pH and volumes are shown in Table 6.2.

Statistical analysis was carried out for each urinary component. Comparisons were made between mean excretions prior to, during and after the ascorbic acid experimental period. In addition, trends observed during the five day period of ascorbic acid ingestion (samples 3, 4 and 5) and the five days post cessation of the protocol (samples 6, 7 and 8) were analysed.

Table 6.2 *Mean excretions of the urinary chemical components (mmol/24 hr), pH and volume (cm³/24 hr) for the 10 participants at different stages during the 12 day experimental period.*

| # | Mg | Ca | Na | K | Vol | pH | Phos | Creat | Cit | Urate | Cl |
|---|------|------|--------|-------|------|------|-------|-------|------|-------|-------|
| 1 | 3.16 | 2.73 | 98.11 | 60.86 | 1457 | 6.23 | 24.34 | 12.09 | 2.17 | 3.15 | 126.7 |
| 2 | 3.04 | 2.36 | 95.59 | 51.83 | 1506 | 6.24 | 26.04 | 12.29 | 2.16 | 3.20 | 120.1 |
| 3 | 4.30 | 2.90 | 108.74 | 65.71 | 1862 | 6.08 | 24.48 | 12.90 | 2.11 | - | 132.3 |
| 4 | 4.09 | 2.73 | 86.30 | 61.05 | 1787 | 6.01 | 24.40 | 12.34 | 2.22 | - | 98.1 |
| 5 | 3.63 | 2.79 | 94.75 | 42.04 | 1586 | 6.03 | 24.94 | 12.49 | 2.06 | 0.72 | 127.0 |
| 6 | 3.60 | 2.86 | 115.29 | 44.33 | 1433 | 6.12 | 20.01 | 11.25 | 1.91 | 1.24 | 124.3 |
| 7 | 3.01 | 2.16 | 61.88 | 33.38 | 1620 | 6.13 | 20.30 | 12.28 | 2.05 | 2.84 | 89.3 |
| 8 | 2.96 | 2.42 | 110.86 | 52.24 | 1552 | 6.20 | 23.18 | 11.72 | 2.13 | 3.04 | 127.7 |

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Probability values for those components in which no significant trends were observed are shown in Table 6.3.

Table 6.3 *Tabulation of probability values of urinary components which showed no specific changes during the 12 day experimental period.*

| Sample No. | Creatinine | Chloride | Citrate | Calcium | Sodium |
|--------------|------------|----------|---------|---------|--------|
| 1,2 vs 3,4,5 | 0.5593 | 0.7440 | 0.8651 | 0.3558 | 0.9839 |
| trend 3-4-5 | 0.6892 | 0.7900 | 0.8632 | 0.8071 | 0.4706 |
| 3,4,5 vs 6 | 0.1165 | 0.7506 | 0.3643 | 0.8869 | 0.2394 |
| trend 6-7-8 | 0.6572 | 0.8690 | 0.4678 | 0.3453 | 0.8245 |
| 1,2 vs 6,7,8 | 0.6393 | 0.4663 | 0.4953 | 0.8150 | 0.9468 |

Probability values of urinary components in which ascorbic acid ingestion resulted in significant changes in urinary composition are listed in Table 6.4. Resultant trends observed in these components are shown graphically in Figures 6.1 to 6.7.

Table 6.4 *Tabulation of probability values of specific urinary components which showed significant changes during the 12 day experimental period.*

| Sample No. | Magnesium | Potassium | Phosphate | Urate | pH | Volume |
|--|-----------|-----------|-----------|---------|---------|---------|
| 1,2 vs 3,4,5 | 0.0050* | 0.9913 | 0.7457 | 0.0400* | 0.0495* | 0.0350* |
| trend 3-4-5 | 0.1696 | 0.0368* | 0.8687 | - | 0.7415 | 0.1504 |
| 3,4,5 vs 6 | 0.3016 | 0.1924 | 0.0465* | 0.6183 | 0.5186 | 0.0482* |
| trend 6-7-8 | 0.2059 | 0.4920 | 0.2717 | 0.0003* | 0.5878 | 0.5463 |
| 1,2 vs 6,7,8 | 0.7751 | 0.0759 | 0.0292* | 0.0060 | 0.3860 | 0.6651 |
| * significantly different at 95 % confidence | | | | | | |

Urate could not be measured in urines where ascorbic acid concentrations were greater than $1136 \mu\text{mol.dm}^{-3}$ [1, 2]. Thus in Table 6.4, the first comparison involving urate is between the means of samples 1 and 2 versus sample 5 and in the second comparison it is between only sample 5 and sample 6.

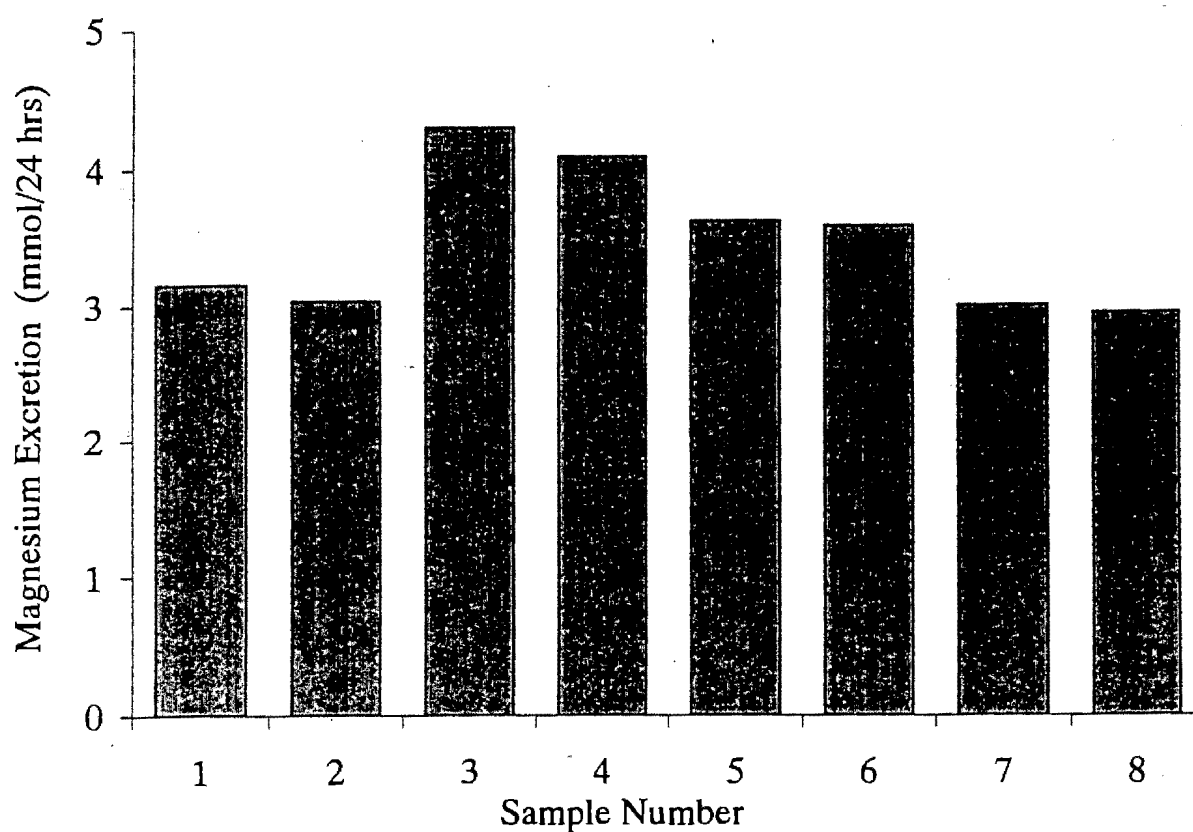


Figure 6.1 *The trend of magnesium excretion (mmol/24 hr) during the experimental procedure.*

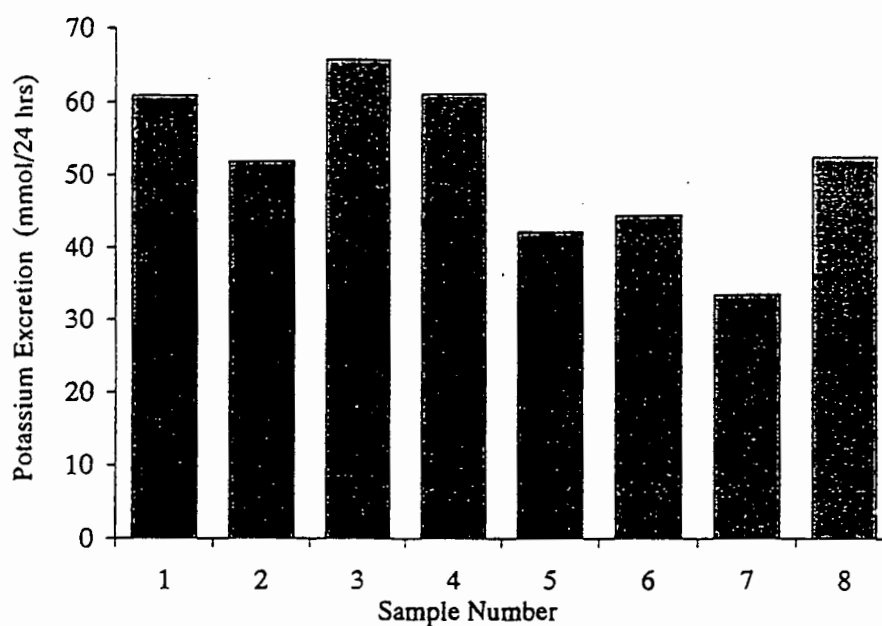


Figure 6.2 *The trend of potassium excretion (mmol/24hr) during the experimental procedure.*

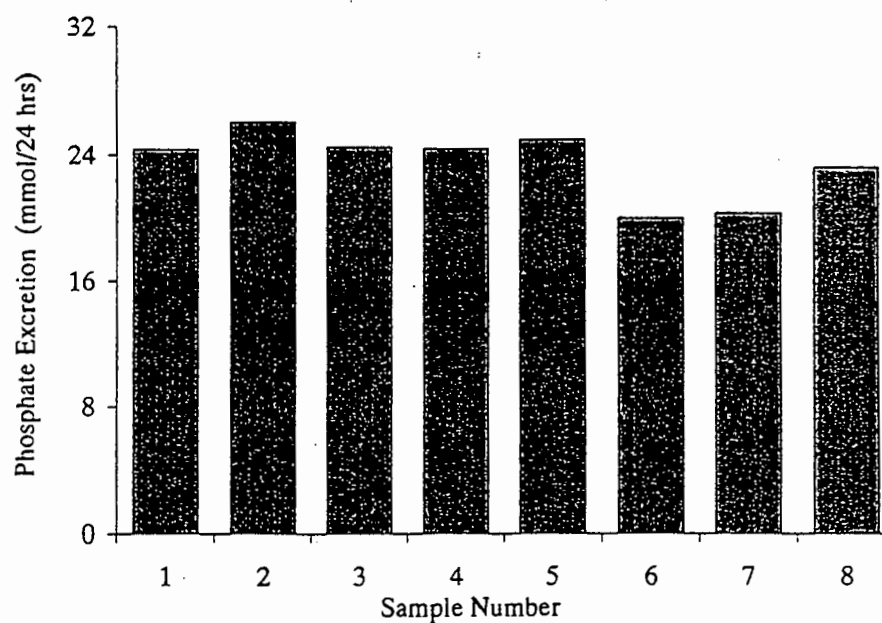


Figure 6.3 *The trend of phosphate excretion (mmol/24hr) during the experimental procedure.*

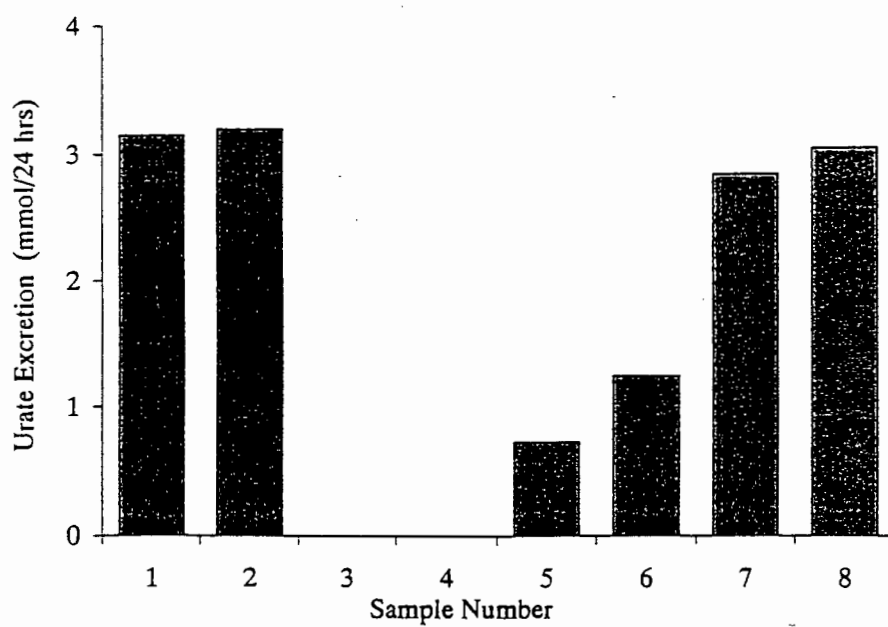


Figure 6.4 *The trend of urate excretion (mmol/24hr) during the experimental procedure.*

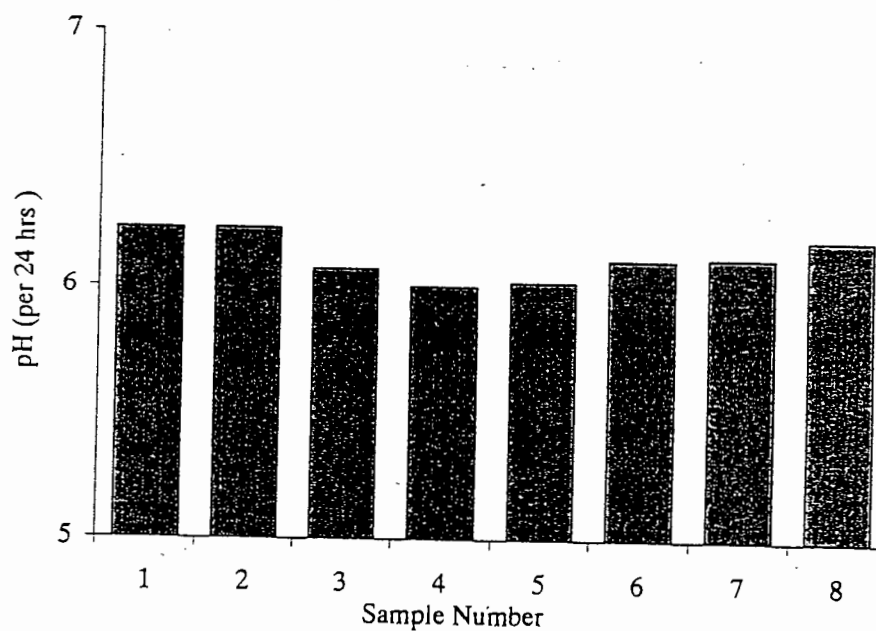


Figure 6.5 *The trend of pH during the experimental procedure.*

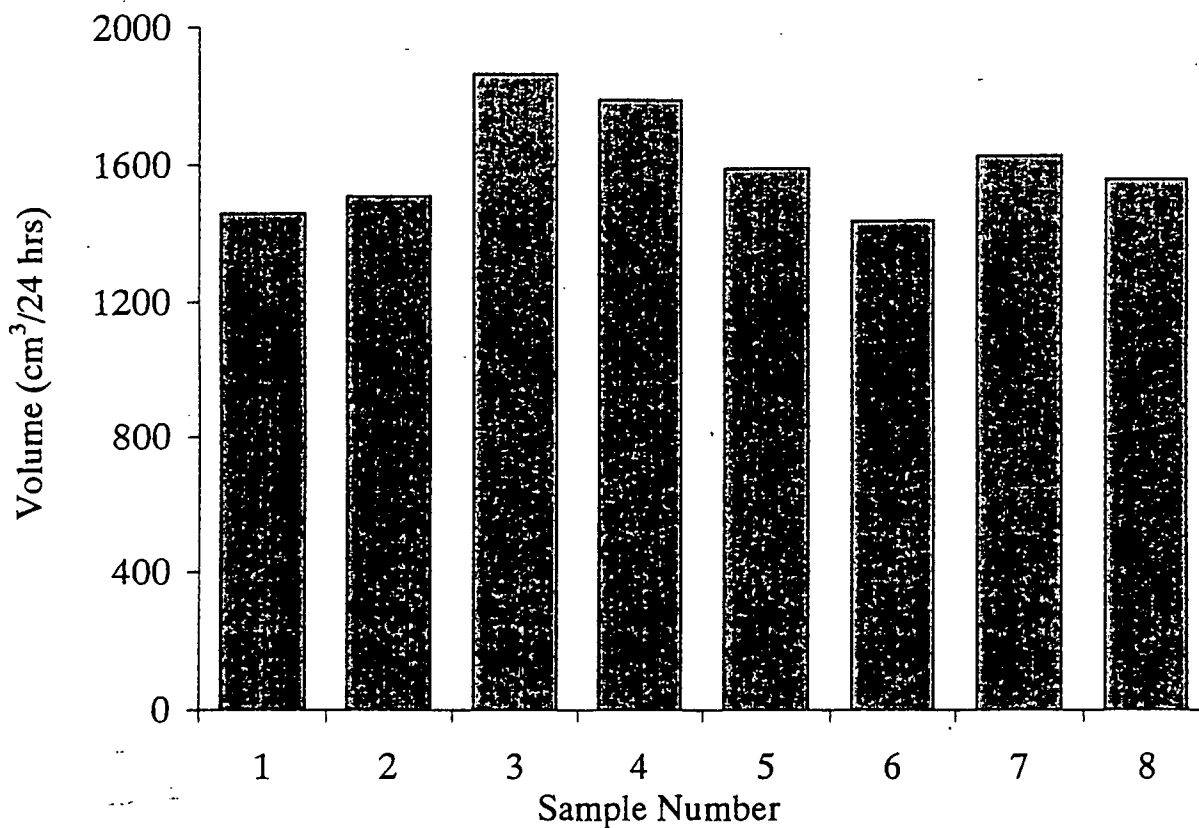


Figure 6.6 *The trend of urinary volume (cm³/24hr) during the experimental procedure.*

6.2 Urinary Oxalate

Table 6.5 describes the manner in which the each of the urine fractions was preserved in the case of urinary oxalate analysis for each urine sample collected.

Table 6.5 *Key to the presence and absence of preservative in the urine sample collections.*

| |
|---|
| a = bulk 24 hour collection (no preservative present) |
| b = representative 24 hour collection + EDTA preservative |
| c = representative 24 hour collection - EDTA preservative |

Ascorbic Acid and Calcium Oxalate Kidney Stone Formation

Urinary oxalate excretion values, determined by enzymatic assay, were measured in the presence and absence of an EDTA preservative (Chapter 3). Table 6.6 shows the mean oxalate excretions for corresponding days in the collection experimental period. Oxalate excretions are shown for the bulk 24 hour collection and the two representative sample collections, in the presence and absence of EDTA.

Table 6.6 *Mean oxalate excretion (mmols/24 hr) values determined for each sample during the experimental period, in the presence and absence of EDTA.*

| Sample Number | Bulk Sample | Representative Sample + EDTA | Representative Sample - EDTA |
|---------------|-------------|---------------------------------|---------------------------------|
| 1 | 0.198 | 0.157 | 0.198 |
| 2 | 0.182 | 0.147 | 0.198 |
| 3 | 0.222 | 0.183 | 0.220 |
| 4 | 0.218 | 0.176 | 0.251 |
| 5 | 0.255 | 0.171 | 0.292 |
| 6 | 0.166 | 0.137 | 0.200 |
| 7 | 0.204 | 0.148 | 0.231 |
| 8 | 0.198 | 0.158 | 0.224 |
| Overall Means | 0.205 | 0.106 | 0.226 |

Figure 6.7 shows a histogram of the mean urinary oxalate excretion determined for each sample during the ascorbic acid experimental period. It is noted that oxalate values in the EDTA preserved samples are lower than those recorded in its absence. (This suggests that

EDTA prevents the *in vitro* conversion of ascorbic acid to oxalic acid which is discussed comprehensively in Section 7.3.2.)

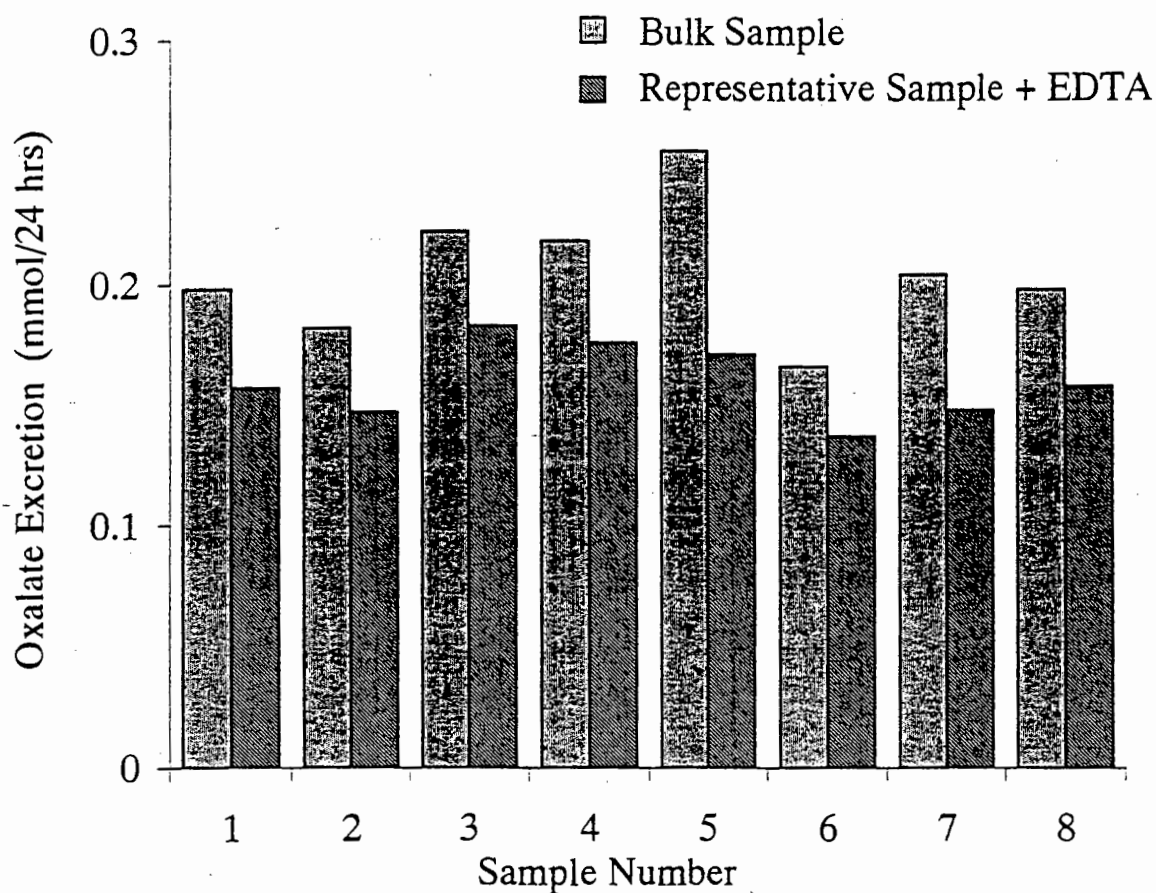


Figure 6.7 Histogram showing preservative effects of EDTA on mean urinary oxalate values and the trend of oxalate excretion (mmol/24hr) during the experimental period.

Table 6.7 shows the probability values for the comparison of mean urinary oxalate values in the presence and absence of EDTA. Probability values show the comparison between “a” and “b”, “a” and “c” and “b” and “c” (e.g. probability for the comparison of “a” and “b” is 0.0015).

Table 6.7 *Probability values for the comparison of urinary oxalate values.*

| Preservative | b | c |
|--|----------|---------|
| a | 0.0015 * | 0.1565 |
| b | - | 0.0001* |
| * significantly different at 95 % confidence | | |
| a : bulk, b : representative + EDTA, c : representative - EDTA | | |

The effect of ascorbic acid ingestion on urinary oxalate was determined for the bulk sample, the representative sample + EDTA and the representative sample - EDTA (Figure 6.7). Statistical analysis was carried out in each case. Table 6.8 shows the probability values obtained in comparing the oxalate concentrations during the different periods of the experimental period.

Table 6.8 *Probability values determined for the comparison of oxalate excretions in preserved and unpreserved urines during different periods of the experimental period.*

| | a | b | c |
|--|----------|---------|----------|
| samples 1,2 vs samples 3,4,5 | 0.0723 | 0.2873 | 0.0093 * |
| trend 3 - 4 - 5 | 0.3587 | > 0.050 | 0.0461 * |
| samples 3,4,5 vs sample 8 | 0.0252 * | 0.1764 | 0.0640 * |
| trend 6 - 7 - 8 | 0.3902 | 0.5659 | 0.5128 |
| samples 1,2 vs samples 6,7,8 | 0.9777 | 0.8534 | 0.3854 |
| * significantly different at 95 % confidence | | | |

6.3 Metastable Limits

The metastable limit for each participant was determined by means of turbidimetry [3, 4] and Coulter multisizer [5, 6, 7, 8, 9, 10]. In each case, equal volumes of filtered urine were dosed with increasing concentrations of sodium oxalate. The turbidity (or particle number) of each consecutive sample was measured and the result plotted against the corresponding sodium oxalate concentration. The MSL was determined by interpolation of the line to the abscissa [10]. The metastable limits measured for each participant are shown in Tables 6.9 and 6.10.

Table 6.9 *Metastable limits (mol of NaOx. dm⁻³) determined by turbidimetry.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 0.10 | 0.09 | 0.04 | 0.04 | 0.09 | 0.20 | 0.15 | 0.06 | 0.04 | 0.04 |
| 2 | 0.10 | 0.11 | 0.07 | 0.03 | 0.12 | 0.18 | 0.20 | - | 0.01 | 0.20 |
| 3 | 0.12 | 0.12 | 0.10 | 0.06 | 0.07 | 0.11 | 0.08 | 0.06 | 0.05 | 0.20 |
| 4 | 0.13 | 0.08 | 0.10 | 0.10 | 0.07 | 0.19 | 0.18 | 0.01 | 0.03 | 0.14 |
| 5 | 0.10 | 0.07 | 0.05 | 0.05 | 0.04 | 0.20 | 0.04 | 0.03 | 0.03 | 0.20 |
| 6 | 0.07 | 0.07 | 0.06 | 0.02 | 0.09 | 0.20 | 0.11 | 0.03 | 0.04 | 0.20 |
| 7 | 0.18 | 0.09 | 0.04 | 0.05 | 0.10 | 0.20 | 0.11 | 0.05 | 0.04 | 0.20 |
| 8 | 0.11 | 0.09 | 0.13 | 0.04 | 0.08 | 0.20 | - | 0.04 | 0.04 | 0.20 |

Table 6.10 *Metastable limits (mol of NaOx. dm⁻³) determined by Coulter multisizer.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 0.10 | - | 0.03 | 0.04 | 0.06 | 0.20 | 0.10 | 0.04 | 0.04 | - |
| 2 | 0.10 | 0.08 | - | 0.01 | 0.12 | 0.17 | 0.20 | - | - | 0.20 |
| 3 | 0.13 | 0.09 | - | 0.04 | 0.05 | 0.11 | - | 0.06 | 0.04 | 0.18 |
| 4 | 0.11 | 0.08 | 0.08 | 0.05 | 0.06 | 0.20 | 0.16 | 0.01 | 0.03 | 0.10 |
| 5 | 0.11 | - | - | 0.04 | 0.02 | 0.20 | 0.05 | 0.03 | 0.02 | 0.16 |
| 6 | 0.08 | 0.05 | 0.06 | 0.01 | 0.07 | 0.20 | 0.09 | 0.03 | 0.04 | 0.17 |
| 7 | 0.17 | 0.09 | 0.04 | 0.07 | 0.11 | 0.20 | - | 0.05 | 0.04 | 0.17 |
| 8 | 0.11 | - | - | 0.04 | - | 0.20 | - | 0.06 | 0.04 | 0.20 |

There were a number of cases in which the metastable limit exceeded the highest prepared sodium oxalate dosing concentration. As a result, it was necessary to group the metastable limit data into three categories in order to carry out a statistical analysis. The categories were :

- (i) less than or equal to a sodium oxalate dose concentration of 0.07 M
- (ii) within the range of 0.07 M to 0.20 M
- (iii) greater than or equal to a sodium oxalate dosing concentration of 0.20 M

The data for turbidimetry and Coulter multisizer were combined into two-way tables (Tables 6.11 and 6.12 respectively). The hypothesis tested in each two way table was whether the proportions of data falling into the three categories of metastable limits was constant over the three categories of the samples. Fisher's exact test was employed to test this hypothesis [11]. The probabilities from the Fisher exact test (turbidimetry (P = 0.798) and Coulter multisizer (P = 0.833)) indicate that the proportions do not change significantly in either determination.

Table 6.11 *Statistical data for the metastable limits determined by turbidimetry.*

| MSL Range (M) | Samples 1,2 | Samples 3,4,5 | Samples 6,7,8 | Totals |
|---------------|-------------|---------------|---------------|-------------|
| ≤ 0.07 | 8 (42.1 %) | 13 (50.0 %) | 13 (44.8 %) | 34 (43.6 %) |
| 0.07 to 0.2 | 8 (42.1 %) | 14 (46.7 %) | 10 (34.5 %) | 32 (41.0%) |
| ≥ 0.2 | 3 (15.8 %) | 3 (10.0 %) | 6 (20.7 %) | 12 (15.4%) |
| Totals | 19 (24.4 %) | 30 (38.5 %) | 29 (37.1 %) | 78 |

Fisher's Exact Test $p = 0.798$ **Table 6.12** *Statistical data for the metastable limits determined by Coulter multisizer.*

| MSL Range (M) | Samples 1,2 | Samples 3,4,5 | Samples 6,7,8 | Totals |
|---------------|-------------|---------------|---------------|-------------|
| ≤ 0.07 | 6 (42.9 %) | 13 (50.0 %) | 13 (52.0 %) | 32 (49.2 %) |
| 0.07 to 0.2 | 6 (42.9 %) | 11 (42.3 %) | 8 (32.0 %) | 25 (38.5 %) |
| ≥ 0.2 | 2 (14.3 %) | 2 (7.70 %) | 4 (16.0 %) | 8 (12.3 %) |
| Totals | 14 (21.5 %) | 26 (40.0 %) | 25 (38.5 %) | 65 |

Fisher's Exact Test $p = 0.833$

6.4 Kinetics of Crystallization

Crystal growth and/or aggregation can be measured by dosing a specific volume of urine with sodium oxalate of concentration equal to the metastable limit (or formation product) concentration and measuring the subsequent increase in turbidity or particle number (volume) as a function of time [10]. A plot of the measurement versus time typically shows an initial

time lag followed by a rising linear portion (Chapter 3), the slope of which is used as an index of the rate of crystallization [10] (Figure 6.8).

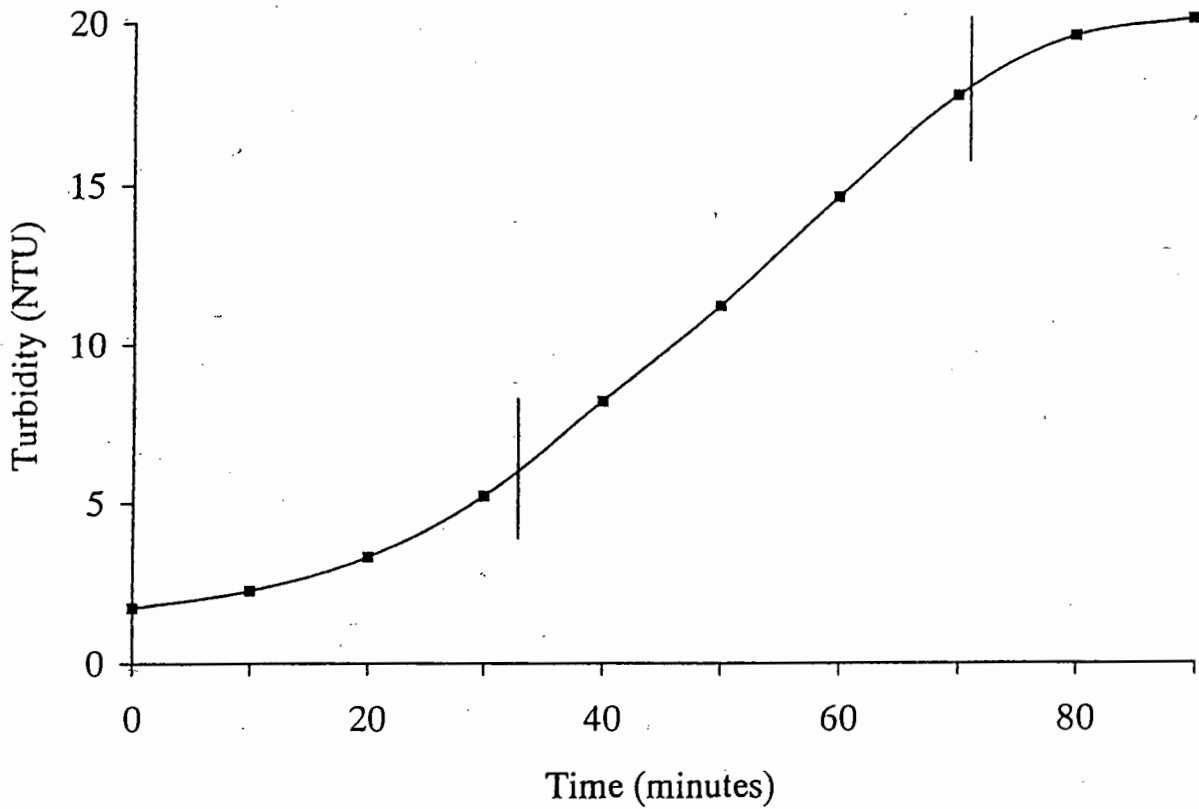


Figure 6.8 Typical plot of crystallization determined by means of turbidimetry. The linear portion indicated on the plot is used as an index of the rate of crystallization.

Table 6.13 shows the calcium oxalate crystallization rate (gradient) determined by turbidimetry for each of the participants at various stages of the experimental period. The crystallization rates determined by means of Coulter multisizer are shown in Table 6.14.

Table 6.13 *Initial calcium oxalate crystallization rates determined at the metastable limit by means of turbidimetry (TU.min⁻¹)*

| | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | 0.594 | 0.472 | 0.423 | 0.250 | 0.380 | ~ 0 | 0.568 | 0.578 | 0.820 | 0.298 |
| 2 | 0.450 | 0.675 | 0.563 | 0.725 | 0.417 | 0.798 | 0.824 | 0.737 | 0.450 | 1.333 |
| 3 | 0.901 | 0.778 | 0.635 | 0.308 | 0.542 | 0.220 | 0.009 | 0.294 | 0.340 | 1.025 |
| 4 | 1.520 | 0.558 | 0.115 | 0.957 | 0.366 | 0.443 | - | 0.090 | 0.260 | - |
| 5 | 4.836 | 0.483 | 0.221 | 0.002 | 0.757 | 0.243 | 0.003 | 0.040 | 0.062 | 1.307 |
| 6 | 0.402 | 0.782 | 0.019 | 0.351 | 0.300 | 0.002 | 0.932 | 0.153 | 0.123 | 0.863 |
| 7 | 1.850 | 0.583 | 0.226 | 0.160 | 0.103 | 0.001 | 0.530 | 0.278 | 0.267 | 1.125 |
| 8 | 1.030 | - | 1.103 | 0.130 | 0.383 | ~ 0 | - | 0.194 | 0.460 | 0.815 |

Table 6.14 *Initial crystallization rates determined at the metastable limit by means of Coulter multisizer (particles.min⁻¹).*

| | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | 372.2 | - | 303.4 | 206.9 | 13.5 | 8.9 | - | 298.5 | 600.7 | - |
| 2 | 220.5 | 169.2 | - | 61.6 | 125.0 | 346.2 | 226.0 | - | - | 235.0 |
| 3 | 234.0 | 78.6 | - | 134.9 | 104.8 | 377.3 | - | - | 303.3 | 269.7 |
| 4 | 425.6 | 336.0 | 92.6 | 311.1 | 20.8 | 391.4 | 505.3 | 5.1 | 522.6 | 401.1 |
| 5 | 423.6 | - | - | 291.7 | 122.6 | 52.8 | 342.4 | - | 244.5 | 398.1 |
| 6 | 333.6 | 347.1 | 334.6 | 196.4 | 133.1 | 18.7 | 476.9 | 442.7 | 306.0 | 146.5 |
| 7 | 479.5 | 387.0 | 372.4 | - | 194.7 | 8.9 | - | 253.9 | 239.4 | 266.7 |
| 8 | 231.9 | - | - | - | - | 1.4 | - | - | 323.4 | 138.7 |

In addition, crystallization rates for each participant were also determined at a "standard" sodium oxalate dosing concentration which was maintained constant throughout the crystallization experiments for a specific participant. The resultant crystallization rates for turbidimetry and Coulter measurements are listed in Table 6.15 and Table 6.16 respectively.

Data collection by turbidimetry and Coulter multisizer for the "standard" sodium oxalate dosing concentrations were incomplete for several reasons. In some cases this was due to "overdosing" (denoted O/S) while in others, equipment failure played a role.

Table 6.15 *Initial calcium oxalate crystallization rates determined after administration of a "standard" sodium oxalate dosing concentration : turbidimetry data (TU.min⁻¹).*

| | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | - | 0.635 | 0.574 | 0.250 | 0.380 | ~ 0 | 0.568 | 0.578 | 0.248 | - |
| 2 | - | 0.675 | 0.563 | o/s | 0.281 | 0.546 | 0.263 | 0.518 | 0.338 | 1.333 |
| 3 | - | 0.361 | 0.635 | 0.093 | o/s | o/s | o/s | 0.075 | 0.006 | 1.025 |
| 4 | - | 0.816 | 0.115 | 0.009 | o/s | 0.443 | - | 0.155 | 0.260 | o/s |
| 5 | - | o/s | o/s | o/s | o/s | 0.243 | o/s | 0.264 | 0.320 | 1.307 |
| 6 | - | o/s | 1.063 | 1.282 | 0.695 | 0.002 | o/s | 0.486 | 0.123 | 1.863 |
| 7 | - | 0.695 | o/s | 0.022 | 0.075 | 0.001 | 1.448 | 0.278 | 0.008 | 1.125 |
| 8 | - | - | 0.357 | 0.130 | 0.830 | ~ 0 | ~ 0 | 0.194 | 0.192 | 0.813 |

* The "standard" dosing concentration was based on the metastable limit concentrations of the two samples collected prior to ascorbic acid ingestion.

Table 6.16 Initial calcium oxalate crystallization rates determined after administration of a "standard" sodium oxalate dosing concentration : Coulter multisizer data (particles.min⁻¹).

| | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | - | - | 303.4 | 206.9 | 13.5 | 8.9 | - | 298.5 | 600.7 | - |
| 2 | - | 30.9 | - | o/s | - | 346.2 | 332.3 | - | - | 235.0 |
| 3 | - | 132.7 | - | 134.9 | 441.9 | o/s | - | o/s | 303.3 | 254.6 |
| 4 | - | o/s | 92.6 | 11.0 | 218.3 | 391.4 | 505.3 | o/s | 613.2 | o/s |
| 5 | - | o/s | - | 291.7 | 397.9 | 52.8 | o/s | - | 511.1 | 398.1 |
| 6 | - | o/s | o/s | o/s | 133.1 | 18.7 | o/s | 668.3 | 304.0 | 163.9 |
| 7 | - | 277.9 | o/s | - | 191.1 | 8.9 | - | 253.9 | 239.4 | 266.7 |
| 8 | - | - | - | - | - | 1.4 | - | o/s | 323.4 | 138.7 |

6.5 Sedimentation of Calcium Oxalate in Urine

In a trial investigation, an attempt was made to determine the rate of aggregation of calcium oxalate monohydrate (COM) crystals using the method developed by Hess *et al.* [12]. However, the results obtained by turbidimetry were erratic. Consequently, a different monitoring method was used in which an aliquot of supersaturated urine was transferred to a spectrophotometer cuvette and absorbance measurements were determined *in lieu* of turbidimetry measurements. The decrease in absorbance is due to the settling of particles, controlled by particle size [12]. Facilities were inadequate to carry out a detailed examination of this method (Chapter 7) and hence results were only obtained in a few urine samples. A plot of absorbance as a function of time (Figure 6.9) illustrates the trend of calcium oxalate crystal sedimentation in the urine of one of the participants in this study. The gradient of the linear portion of the graph is equivalent to the aggregation rate (terminal velocity) of the COM crystals settling in supersaturated urines. The COM aggregation rates presented in Table 6.17

have been determined from 2 urine samples supplied by the same individual. These urine samples (analysis 1 and 2) were collected as “once off” samples in order to test the COM sedimentation experiment and are not associated with the full sample analysis carried out for each participant in the study.

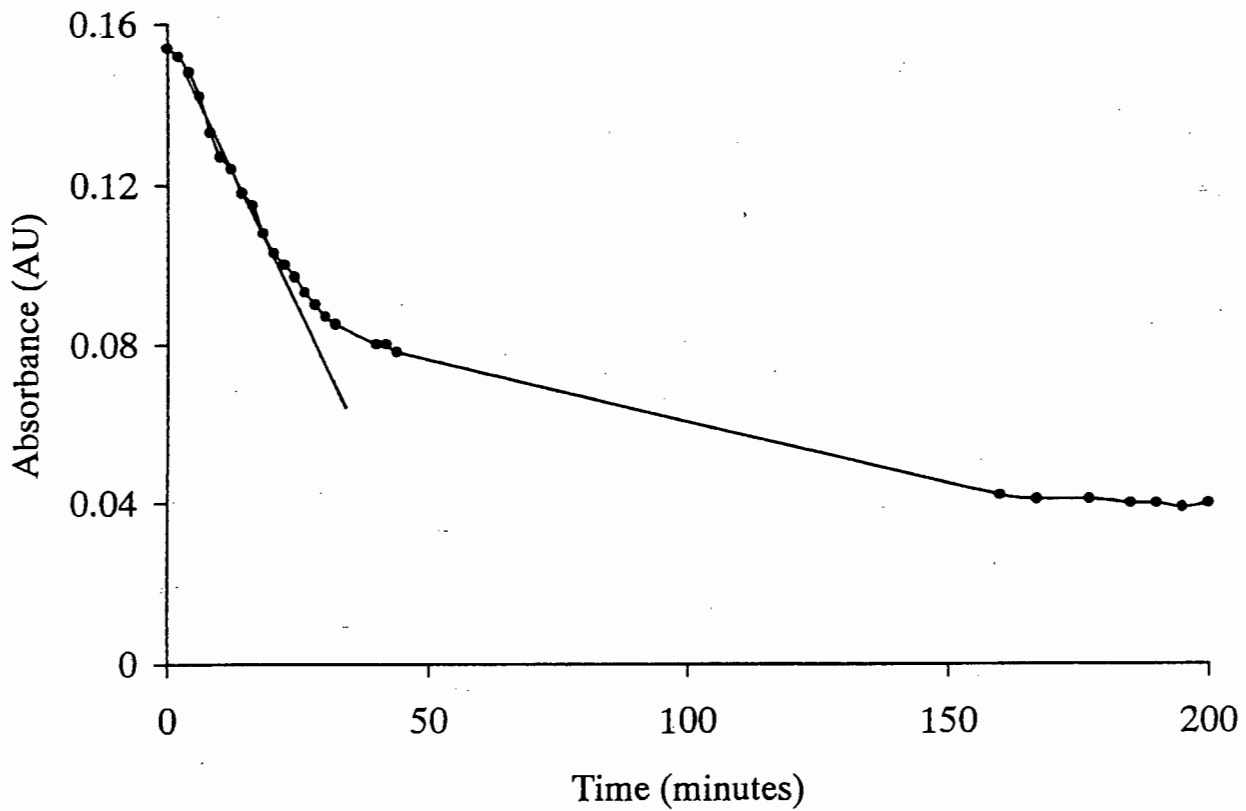


Figure 6.9 A typical experimental plot of absorbance vs time showing the rate of calcium oxalate crystal sedimentation in urine.

Table 6.17 *The crystal aggregation rate of COM crystals in saturated urines with the correlation coefficient corresponding to the linear portion of the plot.*

| Analysis No. | COM Aggregation Rate (s^{-1}) | r |
|--------------|-----------------------------------|--------|
| 1 | 0.27×10^{-2} | 0.9914 |
| 2 | 4.80×10^{-2} | 0.9910 |

6.6 Ascorbic Acid Concentration

Table 6.18 *Key to specific urine voidings used for ascorbic acid analysis during the 24 hour urine collection.*

voiding x = the second voiding during the 24 hour collection period
 voiding y = the fourth voiding during the 24 hour collection period
 voiding z = the final voiding during the 24 hour collection period (i.e. the voiding

Ascorbic acid was determined by FIA using the same chemicals as in the manual titration (Chapter 4). The ascorbic acid analysis by FIA was limited to a maximum concentration of $150 \mu g \cdot cm^{-3}$. After the determination of ascorbic acid by manual titration, the urine sample was diluted to ensure that the ascorbic acid concentration was within this range. The diluted sample was titrated with 2,6 DCPIP and analysed by FIA. This was carried out for each urine voiding for each participant. The results for both methods are shown in Table 6.19.

The table uses the following abbreviations :

1 - 8 : sample number (Table 6.1)

v_x, v_y, v_z : voiding times (Table 6.19)

PT, ES, PR, SH, MG, GV, BM, CW, RG, AM : participants' initials

Table 6.19 *Ascorbic acid concentration determined in diluted urine samples by (a) manual titration (MT) and (b) flow-injection analysis (FIA).*

| | PT | | ES | | PR | | SH | |
|-------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| | MT | FIA | MT | FIA | MT | FIA | MT | FIA |
| #1 v _x | 32.6 | 26.3 | 10 | 13.4 | 51.1 | 46.7 | 46.9 | 122.5 |
| v _y | 58.8 | 54.1 | 10 | 4.4 | 15.9 | 44.5 | 48.6 | 110.5 |
| v _z | 18.6 | 14.6 | 80.8 | 83.2 | 17.3 | 31.3 | 84.6 | 71.4 |
| #2 v _x | 10 | 8.9 | 55.3 | 64.4 | 10 | 13.7 | 59.0 | 60.4 |
| v _y | 10 | 10.8 | 10 | 14.4 | 17.9 | 20.6 | 56.0 | 43.3 |
| v _z | 11.6 | 7.1 | 67.5 | 80.1 | 10 | 4.2 | 25.5 | 50.92 |
| #3 v _x | 51.2 | 52.1 | 39.5 | 46.1 | 10 | 37.9 | 15.1 | 13.7 |
| v _y | 94.6 | 133.6 | 67.9 | 69.2 | 121.6 | 109.9 | 52.0 | 41.9 |
| v _z | 56.8 | 54.0 | 102.5 | 98.5 | 94.1 | 142.1 | 160.3 | 141.0 |
| #4 v _x | 39.8 | 38.3 | 122.7 | 102.1 | 171.5 | 113.8 | 92.5 | 74.8 |
| v _y | 66.8 | 78.2 | 44.7 | 54.4 | 131.7 | 126.8 | 126.1 | 100.0 |
| v _z | 76.7 | 71.4 | 125.5 | 107.5 | 142.5 | 153.1 | 160.2 | 110.1 |
| #5 v _x | 17.3 | 65.9 | 116.4 | 105.5 | 138.5 | 145.9 | 73.8 | 72.8 |
| v _y | 138.1 | 119.6 | 47.1 | 57.1 | 116.5 | 123.7 | 68.0 | 63.9 |
| v _z | 127.4 | 124.4 | 174.8 | 160.8 | 197.9 | 205.3 | 172.8 | 143.5 |
| #6 v _x | 68.1 | 85.3 | 113.3 | 127.8 | 50.3 | 71.9 | 81.5 | - |
| v _y | 67.5 | 92.1 | 127.8 | 165.0 | 112.2 | 104.6 | 100.4 | - |
| v _z | 97.4 | 145.4 | 62.2 | 116.5 | 159.8 | 148.9 | 99.1 | 123.0 |
| #7 v _x | 25.4 | 21.7 | 61.3 | 53.3 | 63.1 | 52.3 | 26.6 | 21.5 |
| v _y | 76.6 | 52.9 | 24.3 | 22.7 | 81.7 | 81.6 | 22.9 | 22.9 |
| v _z | 21.7 | 17.3 | 32.2 | 28.4 | 92.2 | 79.5 | 33.4 | 40.7 |
| #8 v _x | 14.3 | 13.3 | 31.3 | 40.1 | 69.8 | 69.4 | 24.2 | - |
| v _y | 28.5 | 20.1 | 10 | 6.3 | 93.2 | 102.6 | 151.9 | - |
| v _z | 18.6 | 29.0 | 10 | 24.9 | 194.7 | 159.4 | 14.7 | - |

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Table 6.19 *continued*

| | MG | | GV | | BM | | CW | |
|----------|-------|-------|-------|-------|------|------|-------|-------|
| | MT | FIA | MT | FIA | MT | FIA | MT | FIA |
| #1 v_x | 10.0 | 11.7 | - | - | 10.0 | 18.5 | 37.0 | 55.5 |
| v_y | 10.0 | 8.2 | - | - | - | - | 20.3 | 47.3 |
| v_z | 40.0 | 46.0 | 10.0 | 18.9 | 10.0 | 25.3 | 19.7 | 53.4 |
| #2 v_x | 39.5 | 44.9 | 10.0 | 13.1 | 11.5 | 16.7 | 16.0 | 62.4 |
| v_y | 10.0 | 8.5 | 21.5 | 23.4 | 40.7 | 33.1 | 46.4 | 57.9 |
| v_z | 10.0 | 13.7 | 10.0 | 9.9 | 22.1 | 66.7 | 30.2 | 95.8 |
| #3 v_x | 173.4 | 104.3 | 35.9 | 32.6 | 10.0 | 30.5 | 25.7 | 71.4 |
| v_y | 102.8 | 80.9 | 94.7 | 81.1 | 10.0 | 19.9 | 61.7 | 117.7 |
| v_z | 97.5 | 77.8 | 69.5 | 58.2 | 98.2 | 65.1 | 227.9 | - |
| #4 v_x | 102.3 | 86.1 | 80.3 | 90.0 | 92.5 | 73.5 | 78.4 | 40.7 |
| v_y | 64.6 | 71.6 | 91.8 | 96.3 | 80.9 | 72.7 | 138.6 | 99.7 |
| v_z | 84.6 | 87.4 | 91.9 | 92.0 | 87.6 | 73.4 | 133.7 | 73.3 |
| #5 v_x | 103.5 | 75.6 | - | - | 31.9 | 22.6 | 49.0 | 52.7 |
| v_y | 63.8 | 70.8 | 60.3 | 67.5 | 33.5 | 45.2 | 42.9 | 45.2 |
| v_z | 133.9 | 142.8 | 51.2 | 60.0 | 69.0 | 82.7 | 53.1 | 73.0 |
| #6 v_x | - | - | 124.7 | 120.9 | 45.1 | 63.8 | 45.5 | 62.0 |
| v_y | - | - | - | - | 26.4 | 33.7 | 51.0 | 74.7 |
| v_z | 24.7 | 21.5 | 24.1 | 32.0 | 32.4 | 47.5 | 40.8 | 68.5 |
| #7 v_x | 29.6 | 53.0 | 32.7 | 30.0 | 10.0 | 5.6 | 60.6 | 51.9 |
| v_y | 21.5 | 18.1 | - | - | 10.0 | 5.4 | 19.5 | 15.2 |
| v_z | 16.4 | 13.0 | 10.0 | 6.1 | 10.0 | 11.8 | 62.7 | 61.4 |
| #8 v_x | 20.7 | 49.4 | 135.1 | 111.6 | - | - | 55.2 | - |
| v_y | 10.0 | 11.6 | - | - | - | - | 22.1 | - |
| v_z | 12.9 | 20.9 | 66.3 | 79.1 | - | - | 22.2 | - |

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Table 6.19 *continued*

| | RG | | AM | |
|-------------------|-------|-------|-------|-------|
| | MT | FIA | MT | FIA |
| #1 v _x | - | 62.8 | 112.1 | 137.0 |
| v _y | - | 5.7 | 72.8 | 78.9 |
| v _z | - | 24.2 | 86.8 | 178.8 |
| #2 v _x | 27.6 | 57.3 | 113.4 | 155.5 |
| v _y | 19.9 | 43.5 | 106.7 | 142.4 |
| v _z | 10.0 | 23.4 | 43.5 | 79.9 |
| #3 v _x | 82.1 | 122.9 | 52.6 | 47.1 |
| v _y | 53.4 | 89.4 | 84.6 | 132.9 |
| v _z | 85.5 | 146.5 | 64.6 | 108.3 |
| #4 v _x | 119.0 | 93.7 | 89.2 | 78.2 |
| v _y | 93.1 | 84.8 | 86.9 | 80.4 |
| v _z | 69.3 | 58.8 | 48.6 | 46.8 |
| #5 v _x | 75.5 | 67.6 | 68.8 | 54.3 |
| v _y | 161.6 | 136.1 | 58.3 | 47.3 |
| v _z | 87.3 | 77.4 | 45.4 | 45.7 |
| #6 v _x | 49.0 | 49.5 | 34.7 | 45.3 |
| v _y | 72.0 | 76.1 | 69.8 | 76.1 |
| v _z | 96.7 | 91.7 | 10.0 | 7.8 |
| #7 v _x | 10.0 | 29.3 | 10.0 | 11.0 |
| v _y | 45.3 | 62.9 | 10.0 | 16.6 |
| v _z | 25.2 | 50.8 | 10.0 | 19.7 |
| #8 v _x | 29.1 | 60.8 | 10.0 | 20.5 |
| v _y | 45.2 | 86.2 | 10.0 | 17.7 |
| v _z | 10.0 | 19.0 | 10.0 | 9.41 |

The individual ascorbic acid concentrations determined by manual titration at the specified collections during the 24 hour collection period for each of the participants during the

experimental period are given in Appendix D. The mean ascorbic acid concentrations for all participants at each of the above defined voiding times during the 12 day experimental period are given in Table 6.20.

Table 6.20 *Mean ascorbic acid concentrations ($\mu\text{g.cm}^{-3}$) for the three specified voidings determined by manual titration at different stages of the experimental period.*

| Mean ascorbic acid concentration ($\mu\text{g.cm}^{-3}$) for participants | | | |
|---|-------------|-------------|-------------|
| # | Voiding "x" | Voiding "y" | Voiding "z" |
| 1 | 78 | 37 | 26 |
| 2 | 44 | 44 | 26 |
| 3 | 287 | 785 | 1201 |
| 4 | 1034 | 845 | 935 |
| 5 | 863 | 762 | 1132 |
| 6 | 470 | 364 | 201 |
| 7 | 189 | 141 | 92 |
| 8 | 49 | 56 | 24 |

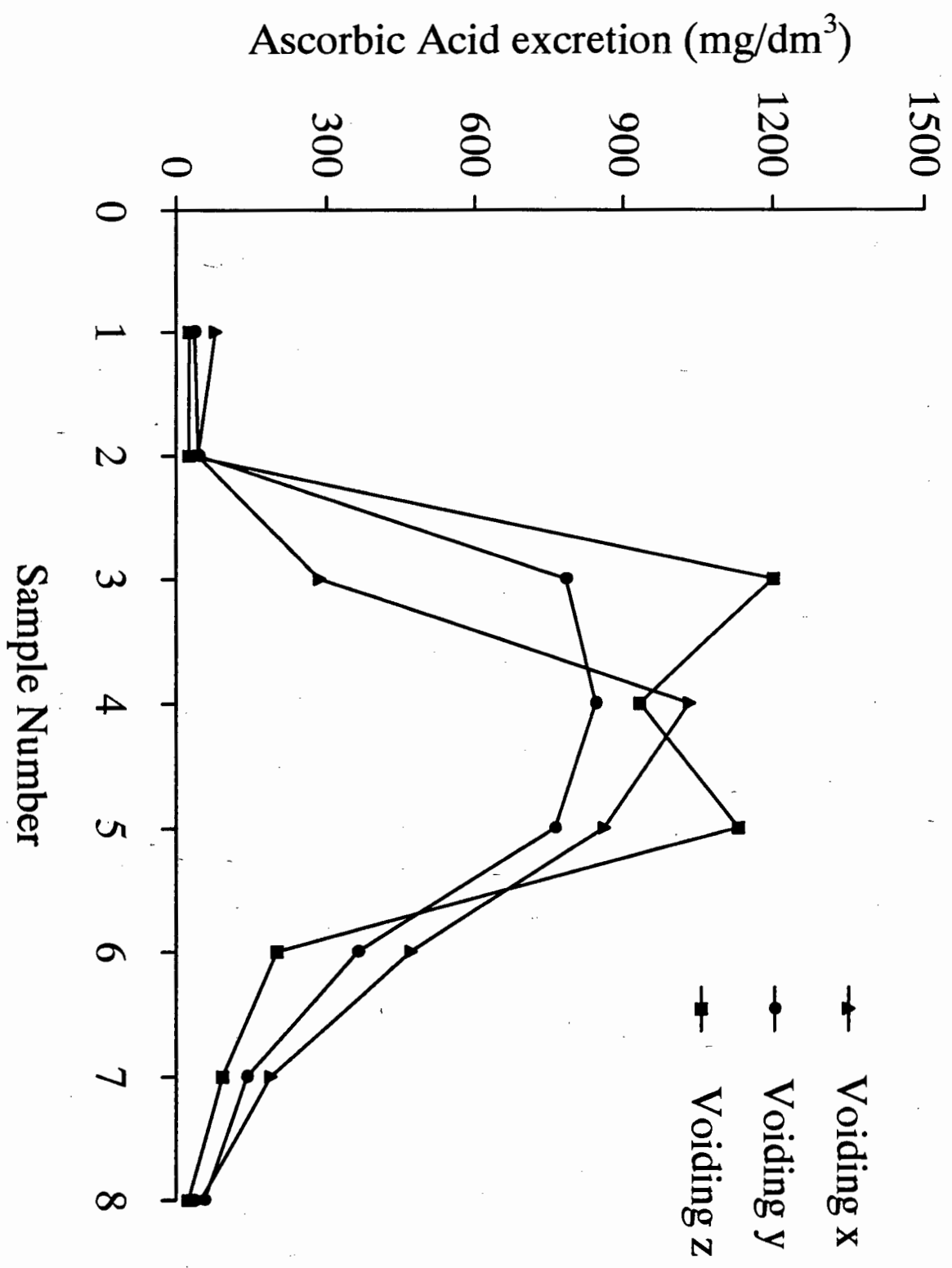
Statistical analysis was carried out to establish the relationship of mean ascorbic acid concentration for each of the three voiding times. The least squares probability values are listed in Table 6.21, showing the comparison between voidings "x" and "y", voidings "x" and "z" and voidings "y" and "z" for each of the sample collections during the experimental period.

Table 6.21 *Probability values determined using least-squares comparing the mean ascorbic acid concentration at each of the voiding times during the experimental period.*

| Sample Number | Probability Values | | |
|--|-------------------------------|-------------------------------|-------------------------------|
| | Voiding "x" vs Voiding "y" | Voiding "x" vs Voiding "z" | Voiding "y" vs Voiding "z" |
| 1 | 0.8313 | 0.7692 | 0.9508 |
| 2 | 0.9956 | 0.9122 | 0.9078 |
| 3 | 0.0023* | 0.0001* | 0.0107* |
| 4 | 0.2448 | 0.5399 | 0.5811 |
| 5 | 0.5472 | 0.1074 | 0.0231 |
| 6 | 0.5479 | 0.1073 | 0.3491 |
| 7 | 0.7737 | 0.5506 | 0.7719 |
| 8 | 0.9662 | 0.8868 | 0.8575 |
| * significantly different at 95 % confidence | | | |

Figure 6.10 shows the trend of the mean ascorbic acid concentration during the 12 day experimental period observed for each of the three voiding times.

Figure 6.10 *Graph showing the trends observed for mean ascorbic acid concentrations during the 12 day experimental period for each of the three urine voidings.*



6.7 Tiselius Risk Index

The values calculated for the Tiselius biochemical risk index [13] for each participant for the corresponding 24 hour collection are presented in Table 6.22.

Table 6.22 *Mean Tiselius risk index of samples during the 12 day experimental period.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | 241.0 | 207.5 | 362.8 | 240.1 | 179.6 | 53.8 | 52.0 | 311.8 | 135.5 | 99.2 |
| 2 | 142.4 | 222.1 | 219.1 | 175.5 | 115.0 | 81.2 | 114.5 | 266.6 | 96.9 | 164.2 |
| 3 | 171.1 | 129.9 | 147.3 | 477.1 | 233.0 | 90.5 | 122.1 | 334.4 | 191.6 | 127.6 |
| 4 | 280.5 | 91.7 | 160.9 | 225.6 | 94.6 | 84.4 | 479.4 | 520.9 | 177.4 | 255.4 |
| 5 | 182.6 | 198.3 | 182.7 | 184.6 | 131.4 | 116.8 | 606.6 | 629.5 | 142.1 | 160.7 |
| 6 | 164.4 | 259.8 | 165.0 | 372.1 | 83.4 | 74.1 | 270.9 | 247.7 | 257.2 | 143.6 |
| 7 | 85.5 | 144.6 | 307.2 | 260.3 | 192.0 | 180.6 | 99.2 | 123.4 | 116.1 | 104.4 |
| 8 | 149.1 | 183.8 | 344.8 | 242.7 | 203.9 | 70.0 | - | 206.1 | 387.5 | 29.9 |

The probability values determined in the comparison of the day-to-day effects on the risk index values of ascorbic acid ingestion during the collection period are shown in Table 6.23. The trend observed in the mean risk index for each of the samples during the ascorbic acid experimental period is shown in Figure 6.11

Table 6.23 *Probability values determined using least-squares comparing the risk index for urine samples collected during the experimental period.*

| Probability values observed for the Tiselius risk index during the experimental period | |
|--|---------|
| samples 1,2 vs samples 3,4,5 | 0.0665* |
| trend samples 3-4-5 | 0.2842 |
| samples 3,4,5 vs sample 6 | 0.4833 |
| trend samples 6-7-8 | 0.9426 |
| samples 1,2 vs samples 6,7,8 | 0.5748 |
| * significantly different at 95 % confidence | |

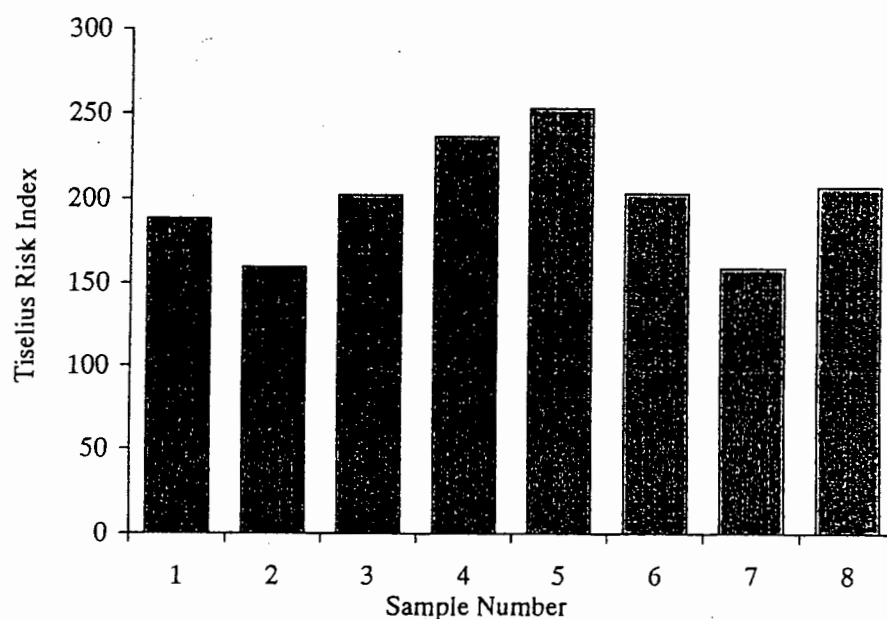


Figure 6.11 *Trend shown by the mean risk index determined for each sample collection during the experimental period.*

6.8 Calcium Oxalate Relative Supersaturation

The calcium oxalate relative supersaturation values [14] for each participant during the experimental period are shown in Table 6.24. The statistical probability values, determined by least-squares, comparing the trends observed during the experimental period are shown in Table 6.25.

Table 6.24 *Mean values for the calcium oxalate relative supersaturation for the samples during the 12 day experimental period.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | 5.307 | 1.913 | 6.151 | 4.787 | 2.884 | 0.544 | 0.389 | 4.363 | 1.564 | 2.257 |
| 2 | 1.964 | 2.314 | 3.089 | 6.113 | 1.731 | 1.634 | 0.316 | 4.145 | 0.781 | 0.436 |
| 3 | 1.630 | 1.647 | 1.400 | 6.244 | 5.088 | 2.141 | 1.489 | 3.921 | 1.569 | 1.103 |
| 4 | 3.893 | 1.489 | 1.469 | 2.389 | 2.550 | 1.850 | 2.700 | 12.28 | 3.113 | 1.262 |
| 5 | 3.063 | 2.450 | 3.200 | 3.522 | 3.280 | 1.708 | 7.877 | 6.714 | 2.227 | 1.707 |
| 6 | 2.741 | 3.537 | 2.102 | 8.988 | 2.962 | 1.199 | 1.590 | 3.020 | 2.584 | 0.385 |
| 7 | 1.279 | 1.874 | 5.478 | 4.662 | 3.855 | 2.527 | 1.139 | 1.543 | 2.020 | 0.545 |
| 8 | 2.323 | 2.572 | 3.378 | 3.629 | 6.639 | 0.821 | - | 1.919 | 1.879 | 0.438 |

Table 6.25 *Probability values comparing the mean calcium oxalate relative supersaturation during the 12 day experimental period.*

| Probability values observed for CaOx Rel. SS during the experimental period | | |
|---|-----------------------------------|-----------------------------------|
| | P ₁ (includes outlier) | P ₂ (excludes outlier) |
| samples 1,2 vs samples 3,4,5 | 0.3128 | 0.5996 |
| trend samples 3-4-5 | 0.2433 | 0.1638 |
| samples 3,4,5 vs sample 6 | 0.7001 | 0.9377 |
| trend samples 6-7-8 | 0.7041 | 0.6644 |
| samples 1,2 vs samples 6,7,8 | 0.9539 | 0.9357 |

6.9 References

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Chapter 7.

Discussion and Conclusions

Several variables were measured in each of the urines collected by each of the ten participants. In addition, individual urines were divided into fractions for various analyses (e.g. effect of an EDTA preservative, oxalate and ascorbate determinations). As such, many comparisons need to be made and many possible trends need to be investigated. The approach which follows is to introduce meaningful points for discussion by means of appropriate questions which will focus attention on key issues relating to the role of ascorbic acid in calcium oxalate kidney stone formation.

7.1 Analysis of creatinine, citrate, chloride, sodium and calcium

All urinary components were determined in each urine sample. Despite collection in the presence and absence of the EDTA preservative, the urinary components discussed in this and the following section were analysed in the absence of EDTA. Mean values for each component analysed in the "bulk" 24 hour urine collection are detailed in Table 6.2. The following sub-sections discuss the components which did not change significantly during the 12 day experimental period.

7.1.1 Is there a significant change in the urinary component excretion after ingestion of ascorbic acid ?

No significant difference was observed in the excretion of creatinine, chloride, citrate, calcium or sodium in urine samples collected during ascorbic acid ingestion (samples 3, 4 and 5) relative to values prior to commencement of the protocol (samples 1 and 2) (Table 6.3).

Hence, dietary ascorbic acid has no significant effect on the excretion of any of the aforementioned urinary components.

7.1.2 Is there a significant change in the excretion of the urinary components during the protocol itself, i.e. as the ingestion of ascorbic acid proceeds ?

No significant increase or decrease occurred in urinary creatinine, chloride, citrate, calcium or sodium during the ascorbic acid protocol (trend 3-4-5, Table 6.3). Thus, prolonged periods of ascorbic acid intake have no effect on the aforementioned chemical components excreted in the urine.

7.1.3 Is there a significant change in the excretion of the urinary components immediately post cessation of the protocol ?

There is no significant change in the urinary creatinine, chloride, citrate, calcium or sodium excretion immediately post cessation ascorbic acid ingestion (3, 4, 5 vs 6, Table 6.3). Thus, cessation of ascorbic acid ingestion has no effect on the urinary components.

7.1.4 Is there a significant change in the excretion of the urinary components during the 5 day period following cessation of ascorbic acid ingestion ?

No significant changes occur in any of the urinary components during this period (trend 6-7-8, Table 6.3).

7.1.5 Is there a difference between pre-protocol and post-protocol values ?

Table 6.3 shows that the values after cessation of the protocol (samples 6, 7 and 8) are not significantly different to control values (samples 1 and 2) indicating that ascorbic acid ingestion has no effect, delayed or otherwise, on the excretion of these components.

7.1.6 Comments

Megadose ascorbic acid ingestion has not been reported to have had any effects on creatinine, chloride, calcium or sodium levels excreted in the urine. The results of the present study are thus in agreement with other studies with respect to these components.

The results of the present study indicate that there is no significant effect on citrate excreted in the urine, despite the megadose ingestion of ascorbic acid for an extended period. Previous literature has indicated an impairment of ascorbic acid absorption and a decreased citrate and ascorbate excretion in the urine of stone forming patients [1]. The results obtained in the latter study show that ascorbic acid and citrate, both of which are hydrocarboxylic acids, share a common carrier in the human gut which is possibly defective in stone forming subjects. As a result, stone formers show decreased citrate and increased oxalate and calcium excretions. Results of the investigation showed that citrate depressed ascorbate absorption from the gut into the blood. The inability of ascorbate to inhibit citrate uptake is consistent with a greater affinity of the carrier for citrate. The inhibition of citrate on the uptake of ascorbic acid has significant bearing on calcium stone disease, since in stone formers the malabsorption of ascorbate will be potentiated by the presence of citrate [1]. These interactions result in an increase in the amount of ascorbate available for conversion to oxalate in the gut and may subsequently result in hyperoxaluria.

The finding in the present study that citrate levels are unaffected by ascorbic acid ingestion suggests that normal individuals ingesting high doses of ascorbic acid are not exposed to competition for a carrier in the gut. Since ascorbic acid does not significantly affect

citrate levels, the possibility of hyperoxaluria due to lower levels of this component is diminished.

It is of interest to note that the citrate excretion levels in the special case (Chapter 5) were not significantly different to those observed in the other participants in this investigation. Nor did the levels fluctuate significantly during the course of the 16 day protocol. It is thus concluded that ascorbic acid and citrate do not interact and compete for a carrier in the gut in normal individuals nor in the case of the single potential stone former analysed in this study.

7.2 Analysis of magnesium, potassium, phosphate, urate, pH and volume

Significant changes in the concentration of some of the urinary components were observed during the 12 day investigation. These changes were ascertained statistically by comparing the mean excretion, for all 10 participants, of a particular urinary component during a 24 hour collection period with the mean excretion of the same urinary component during other 24 hour collection periods. Significant changes were observed during the experimental period for magnesium (Figure 6.1), potassium (Figure 6.2), phosphate (Figure 6.3) and urate (Figure 6.4) as well as for pH (Figure 6.5) and volume (Figure 6.6). The mean excretion and probability values for each of the components are shown in Table 6.2 and 6.4 respectively.

As discussed in Section 3.3, determination of urate concentration in the presence of very high ascorbic acid concentrations is not possible due to masking effects of the latter. The results obtained for urate excretion levels are thus speculative.

7.2.1 *Is there a significant change in the urinary component excretion after ingestion of ascorbic acid ?*

There is a significant increase in magnesium excretion during the period of ascorbic acid ingestion ($p = 0.0050$, Tables 6.2 and 6.4). Since magnesium is a low molecular weight

inhibitor in urine, an increase in the excretion of this component will result in a reduced risk in the stone forming potential of the urine [2, 3, 4]. Thus with regard to this parameter, it would seem that ingestion of ascorbic acid reduces the risk of stone formation.

No significant changes were observed in the excretion of urinary potassium ($p = 0.9913$) and phosphate ($p = 0.7457$) during the period of ascorbic acid ingestion relative to values prior to commencement of the protocol (samples 1 and 2 vs samples 3, 4 and 5, Table 6.4).

A significant decrease in the urate excretion is observed between the two samples collected prior to ascorbic acid ingestion and the sample collected on the fifth day of ascorbic acid ingestion period ($p = 0.040$).

A significant decrease ($p = 0.0495$) is observed in the pH of the urines after commencement of the protocol (samples 1 and 2 vs samples 3, 4 and 5, Table 6.4). This result is not surprising since the ascorbic acid is acidic by nature and hence excessively high ingestion of the vitamin would result in a decrease in pH. Since the solubility of calcium oxalate in urine increases at lower pH values, ingestion of ascorbic acid would decrease the risk of stone formation.

Urine volumes showed a significant increase ($p = 0.0350$) in sample collections during the 5 day ascorbic acid ingestion period relative to control collections (Table 6.4). The increase in urinary volume is probably due to the increase in fluid intake required for ingestion of ascorbic acid tablets four times during the 24 hour period. Although participants were encouraged to consume extra fluid during the control collections, it appears that they failed to do so. It is therefore retrospectively concluded that a placebo should have been administered during the control collections.

7.2.2 Is there a significant change in the excretion of the urinary components during the protocol itself, i.e. as the ingestion of ascorbic acid proceeds ?

No significant change is observed in the magnesium ($p = 0.1696$) or phosphate ($p = 0.8687$) during the period of ascorbic acid ingestion. Thus, although there was a significant increase in magnesium excretion when the protocol commenced, it does not continue with prolonged ingestion of ascorbic acid.

A significant decrease ($p = 0.0368$) in urinary potassium excretion occurs during the 5 day period of ascorbic acid ingestion (Table 6.4). Prolonged ascorbic acid ingestion causes a decrease in potassium excreted in the urine. Studies have shown that potassium depletion can decrease urinary citrate [5] but can increase urinary calcium excretion [6]. In the present study, neither of these two urinary components were affected. Nevertheless, the reported effects on citrate and calcium both represent an increasing risk of calcium oxalate stone formation and therefore the depleted urinary potassium levels are noteworthy.

No significant changes in urinary pH ($p = 0.7415$) and volume ($p = 0.1504$) are observed during the period of ascorbic acid ingestion. Therefore, once the urine has been acidified by the initial ingestion of ascorbic acid, further lowering of urinary pH does not occur during the course of further ascorbic acid ingestion. This is expected, since once the body has absorbed the necessary ascorbic acid to saturate the body pool [7, 8], excess amounts are excreted unchanged in the urine [9]. Urinary volumes did not change significantly. Since ascorbic acid tablets were ingested four times daily, participants consumed a constant intake of fluid during the 5 day period.

7.2.3 Is there a significant change in the excretion of the urinary components immediately post cessation of the protocol ?

The magnesium excretion during the 24 hour period immediately post cessation of ascorbic acid ingestion is not significantly different to the mean magnesium excretion during the ascorbic acid ingestion period ($p = 0.3016$). Thus, the original increase in urinary magnesium observed during ascorbic acid ingestion is maintained as would be the inhibitory activity.

A significant difference is not observed in the urinary potassium excretion ($p = 0.1924$) for samples collected during the 24 hour period post cessation of the protocol, relative to the protocol itself (samples 3, 4 and 5 vs sample 6, Table 6.4). The significant decrease observed in potassium excretion during the 5 day ascorbic acid ingestion period is thus maintained during the first 24 hour period post cessation of ascorbic acid ingestion.

A significant decrease in the urinary phosphate ($p = 0.0465$) excreted in the urine is observed in the 24 hour period immediately post cessation of ascorbic acid ingestion. Low urinary phosphate excretion with concomitantly high calcium excretion has been observed in previous work [10] with the result that urinary phosphate concentration has been linked with the risk of stone formation. A further study has shown that phosphate may play a role in the pathogenesis of urinary calculi [11]. In the present study, the urinary calcium levels were unaffected during the experimental period. However, the reported effect on urinary phosphate represents an increasing risk of calcium oxalate stone formation and therefore depleted levels of phosphate are noteworthy.

A significant difference was not observed in the urate excretion ($p = 0.6183$) post cessation of ascorbic acid ingestion.

A significant change in urinary pH is not observed during the 24 hour collection period immediately post cessation of ascorbic acid ingestion ($p = 0.5186$) indicating that the acidifying effect previously described is maintained.

A significant decrease in urinary volume ($p = 0.0482$) occurs during the 24 hour period immediately post cessation of ascorbic acid ingestion. As discussed earlier, this decrease in urinary volume is probably due to the suspension of the protocol in which ascorbic acid was taken, with water, 4 times in 24 hours.

7.2.4 Is there a significant change in the excretion of the urinary components during the 5 day period following cessation of ascorbic acid ingestion ?

The decreasing trend in magnesium excretion exhibited by samples 3, 4 and 5 is continued by samples 6, 7 and 8 (Table 6.2) but is not significant ($p = 0.2059$). Inspection of the values in Table 6.2 suggests that magnesium excretion gradually returns to its pre-protocol level.

No significant change occurs in the excretion of urinary potassium ($p = 0.4920$) or urinary phosphate ($p = 0.2717$) during the 5 day period post cessation of ascorbic acid ingestion. Hence, the effects of the ascorbic acid ingestion which resulted in a decrease in urinary potassium carry over into the period after cessation of ascorbic acid ingestion i.e. ascorbic acid has long term effects on the urinary potassium excretion (Section 7.2.5). Although there is a significant decrease in urinary phosphate immediately post ascorbic acid cessation, no further decreases are observed.

There is a significant linear increase in urate ($p = 0.0003$) excreted in the urines collected during the post protocol period.

Urinary pH ($p = 0.5878$) and volume ($p = 0.5463$) do not change significantly during the five day post ascorbic acid collection period (Table 6.2), suggesting that there are no long term effects on the urinary pH or volume after megadose ingestion of ascorbic acid.

7.2.5 Is there a difference between pre-protocol and post-protocol values ?

Table 6.4 shows that the magnesium values after cessation of the protocol are not significantly different to control values. Thus, although ascorbic acid ingestion produced an initial increase in magnesium excretion, which was maintained throughout the protocol, values returned to control levels after cessation of the protocol. This indicates that ascorbic acid has no long term or delayed effects on the excretion of magnesium.

Potassium values after the cessation of ascorbic acid ingestion are marginally lower than control values indicating that the decreasing trend observed during the protocol has a "carry-over" effect. However, the potassium excretion for sample 8 (Table 6.2) suggests that the values would probably return to control values within a few more days.

Pre- and post- protocol values for phosphate are significantly different ($p = 0.0292$, Table 6.4). The significant decrease in urinary phosphate excretion which occurred immediately post cessation of ascorbic acid is thus retained.

Although urate excretion continues to be significantly depressed in samples 6, 7 and 8 relative to control values 1 and 2, the mean value for sample 8 appears to be approaching pre-protocol values.

Finally, Table 6.4 shows that pH and volume values have returned to their control values.

7.2.6 What are the overall trends and effects of ascorbic acid ingestion on potassium, phosphate, magnesium, urate, pH and volume ?

A significant increase in magnesium excretion occurs after ascorbic acid ingestion commences. However, prolonged ingestion of ascorbic acid does not result in a continued increase in magnesium levels - the trend observed during the five day ascorbic acid ingestion

period shows no significant change. In the period post cessation of ascorbic acid ingestion, a noticeable (but not significant) decrease in urinary magnesium is observed suggesting that in the absence of the ascorbic acid, magnesium excretion returns to control levels. Thus, with respect to this risk factor, it appears that ascorbic acid ingestion reduces the stone forming potential of the urine, *but only while the ingestion continues*. As soon as the ingestion ceases, the effect weakens.

Ascorbic acid ingestion does not have an immediate effect on the levels of potassium excreted in the urine. However, urinary potassium does decrease significantly during prolonged ascorbic acid ingestion. The results of the present study indicate that continued ingestion of ascorbic acid may cause long term effects in the excretion of this component. Because potassium depletion is associated with hypocitraturia [5] and raised calcium excretion [6], it is concluded that ascorbic acid ingestion, if continued long term may increase the risk of calcium oxalate stone formation.

While there were no significant differences in phosphate excretion immediately after the commencement of ingestion of ascorbic acid and during the protocol itself, a significant decrease occurred in the excretion of this component in the 24 hour period immediately post cessation of ascorbic acid ingestion. This suggests that ascorbic acid may have some sort of delayed effect on urinary phosphate in which a decrease in the excretion of this component occurs. Despite the increasing trend in the phosphate concentrations during the five day period post ascorbic acid cessation, the increase is not significant. Control values (samples 1 and 2) are significantly raised compared to the urinary phosphate levels determined during the post protocol period. Thus the effect of ascorbic acid on phosphate excretion, may be long term. Decreased phosphate excretion is associated with hypocalciuria [10, 11] and hence long term ingestion of ascorbic acid may increase the risk of calcium oxalate stone formation.

Although urate excretion trends have been suggested, results must be regarded as speculative as previously described (Section 3.3).

Ascorbic acid ingestion causes a significant decrease in urinary pH. However, the effect appears to be short term as values return to their control values after the protocol is suspended (Table 6.2 and 6.4).

During the ingestion of ascorbic acid the urinary volume increased significantly relative to control sample collections. After the ingestion of ascorbic acid had ceased, there was a significant decrease in the urinary volumes. These trends indicate that urinary volume was probably dependent on additional fluid intake for the ingestion of ascorbic acid tablets during the five day ascorbic acid ingestion period.

7.3 Oxalate Analysis

Urinary oxalate is an essential parameter in the assessment of the stone forming potential of the urine. Mean urinary oxalate excretion values and the relevant statistical details are shown in Tables 6.6, 6.7 and 6.8. The effect of ascorbic acid and the relevance of EDTA as a preservative to prevent the conversion of ascorbic acid to oxalic acid are discussed in the following sub-sections.

7.3.1 Is there a significant difference between oxalate values in the unpreserved urine samples (i.e. between the bulk sample and the representative sample - EDTA) ?

Tables 6.6 and 6.7 shows that there is no significant difference ($p = 0.1565$) when the respective means for the oxalate excretion in the “bulk” (0.205 mmol/24 hr) and the “- EDTA” (0.226 mmol/24 hr) samples are compared. It is thus concluded that the “- EDTA” sample is truly representative of the “bulk” sample.

7.3.2 *Is there a significant difference between the oxalate values in the preserved representative 24 hour sample (“+ EDTA”) and the unpreserved samples (“bulk” and “- EDTA”) ?*

Tables 6.6 and 6.7 shows that significant differences exist between the preserved sample (mean value = 0.160 mmol/24 hr) and the “bulk” sample (mean value = 0.205 mmol/24 hr) ($p = 0.0015$) and between the preserved sample and the “- EDTA” sample (mean value = 0.226 mmol/24 hr) ($p = 0.0001$).

Since the unpreserved samples have higher oxalate values (Table 6.6), it is concluded that *in vitro* conversion of ascorbic acid to oxalic acid is occurring in these samples but the conversion is prevented in the presence of EDTA. These results therefore demonstrate the need for such a preservative when analysing for oxalate in the presence of ascorbate. Thus, the oxalate values in the present study which have been determined in the presence of EDTA are not erroneously high (due to *in vitro* conversion of ascorbate) and can be regarded with confidence.

7.3.3 *How do the preservatives affect each sample and the analysis thereof ?*

There is no significant difference ($p = 0.9874$) in the trends for each of the collections (with and without preservative) collected during the 12 day experimental period (Figure 6.7). Thus, since the trend for the “bulk”, “+ EDTA” and “- EDTA” collections are the same, it can be concluded that the preservative did not interfere with the analysis. Both “+ EDTA” and “- EDTA” samples can therefore be regarded as true representations of the 24 hour urine collected as far as the trends are concerned. However, accurate, quantitative, oxalate values can be obtained only from the “+ EDTA” sample.

7.3.4 *Is there a significant change in oxalate excretion after ingestion of ascorbic acid ?*

One of the participants showed extremely high oxalate concentrations in the “bulk” and the “- EDTA” urine samples on day 7 of the protocol, which are outliers. This might indicate that in this participant, *in vitro* conversion of ascorbate to oxalate occurred to a very large extent, more so than in other participants. The outliers were thus considered as true values of oxalate excretion in this individual and statistical analysis was carried out in the presence of the outlying values.

A comparison between the mean oxalate excretion for the controls (samples 1 and 2) and the mean oxalate excretions during the 5 day period of ascorbic acid ingestion (samples 3, 4 and 5) in the “bulk” and “+ EDTA” samples indicates no significant difference ($p = 0.0723$ and 0.2873 respectively, Table 6.8). Thus, it is concluded that ascorbic acid ingestion does not result in an immediate *in vivo* conversion to oxalate. (The significantly higher oxalate in the “- EDTA” sample ($p = 0.0093$) confirms the *in vitro* conversion of ascorbate to oxalate discussed earlier).

7.3.5 *Is there a significant change in oxalate excretion during the protocol itself, i.e. as the ingestion of ascorbic acid proceeds ?*

There is no significant change in urinary oxalate excretion in the EDTA preserved sample ($p > 0.05$) or in the “bulk” sample ($p = 0.3587$) during the 5 days of ascorbic acid ingestion (3-4-5, Table 6.8). This confirms the results of Hughes *et al.* [12] who assumed that there is a rate limiting step in the absorption and excretion of urinary oxalate, resulting in a plateau effect in the oxalate excretion. Thus, in the present study, continued ascorbic acid intake does not cause an increase in oxalate excretion. (The significant increase in oxalate excretion observed in the “- EDTA” sample ($p = 0.0461$) once again demonstrates *in vitro* conversion of ascorbic acid to oxalic acid).

7.3.6 Is there a significant change in urinary oxalate excretion after cessation of the ascorbic acid protocol ?

No significant change in the "+ EDTA" and "- EDTA" fractions ($p = 0.1764$ and 0.0640 respectively, Table 6.8) is observed in the mean oxalate excretion when the values obtained during the ascorbic acid protocol are compared to values obtained after its suspension. However, the significant increase ($p = 0.0252$) in oxalate excretion observed in the "bulk" sample is due to *in vitro* conversion of ascorbate to oxalate. Thus, it can be concluded that oxalate excretion is not affected after ascorbic acid ingestion has been halted.

7.3.7 Is there a significant change in urinary oxalate excretion during the 5 day period post cessation of ascorbic acid ingestion ?

Table 6.8 shows that no significant difference occurred in the trend of oxalate excretion in samples 6, 7 and 8. This is contrary to the findings of Hatch *et al.* [13] who demonstrated that there may be retention of urinary oxalate which only manifests itself after a period in which no ascorbic acid is ingested.

7.3.8 Is there a difference between pre- protocol and post- protocol values ?

Table 6.8 shows that the values after the cessation of the protocol (samples 6, 7 and 8) are not significantly different to control values (samples 1 and 2) indicating that ascorbic acid ingestion does not have a delayed effect on oxalate excretion.

7.3.9 Comments

An EDTA preservative is necessary to prevent the *in vitro* conversion of ascorbic acid to oxalic acid. This precaution prevents erroneously high urinary oxalate concentrations being determined and supports the findings of several other workers [12, 13, 14, 15].

The presence or absence of a preservative in the urine collections does not affect the determination of excretion trends i.e. observed trends in preserved and unpreserved samples are consistent and do not show any significant differences. Representative samples are no different to the bulk 24 hour collection with respect to the trends and hence can be considered as being truly representative of the 24 hour collection. Therefore, data collected for the samples containing EDTA are indeed "representative" of the "bulk" sample and can be used for the determination of both qualitative and quantitative results.

The present study has demonstrated that megadose ingestion of ascorbic acid has no effect, delayed or otherwise, on the excretion of urinary oxalate. This is in agreement with several studies [12, 16, 17, 18, 19] but in conflict with others [13, 14, 20, 21, 22, 23].

7.4 Metastable Limits

As explained in Section 6.3, metastable limit (MSL) data were combined to form two-way tables (Table 6.11 and 6.12). The hypothesis tested in the two way tables was whether the proportions of data falling into the three categories (Section 6.3) are constant. Fischer's exact test was employed to test this hypothesis in each of the tables for the MSL's determined by turbidimetry and Coulter multisizer.

The probabilities from the Fischer exact test indicate that the proportions do not change significantly in either determination (turbidimetry : $p = 0.798$ and Coulter multisizer : $p = 0.833$). Since the proportions obtained in the analysis were consistent it was concluded that a more complex analysis was unwarranted. Thus no particular trends were evident in the MSL's obtained by the two techniques in this study (Table 6.9 and Table 6.10). The absence of a definite trend suggests that megadose ingestion of ascorbic acid does not influence the

nucleating potential of the urine. This supports the findings of several other workers who have reported that ascorbic acid does not influence the formation of kidney stones [12, 17, 18, 24].

7.5 Kinetics of Crystallization

The metastable limit of a urine, as measured in this study, indicates the level of sodium oxalate required to induce crystallization with respect to calcium oxalate. At supersaturation, calcium oxalate crystal nucleation no longer occurs. Instead, calcium oxalate crystal growth and/or aggregation take place. These mechanisms can be monitored by measuring the response of the urine to a fixed sodium oxalate load equivalent to or above the metastable limit [25]. This is achieved by measuring the increase in turbidity or particle number as a function of time. A typical plot (obtained in experimental analysis in the present study) of this relationship has been shown previously in Figure 6.8. The initial time lag is followed by a linear portion, the gradient of which is a measure of the rate of crystallization (growth or aggregation or both).

Calcium oxalate crystal growth rates, measured for each of the ten participants by turbidimetry and Coulter multisizer, are shown in Tables 6.13 and 6.14 respectively. The crystallization rates in both tables were determined at the sodium oxalate dosing concentration which corresponded most closely with the experimentally determined MSL. In some cases, kinetic data could not be obtained by Coulter multisizer due to maintenance requirements of the instrument.

Although crystallization rates were determined under the same experimental conditions for each urine sample, results obtained by both techniques showed no specific trend during the twelve day experimental period. Statistical analysis was not attempted. This indicates that despite megadose ingestion of the vitamin, ascorbic acid has no obvious effect on the growth and aggregation of calcium oxalate crystals in urine. Once again, this supports the results of other studies which indicate that ascorbic acid does not influence the formation of kidney stones [12, 16, 17, 18, 24]. Since no trends were established in the rate of crystallization in urine, no generalisations can be made. Some individuals might not be able to handle

megadoses of ascorbic acid and will show high crystallization rates which may promote stone formation, whereas in other cases the ascorbic acid ingestion may have no effect.

In addition to dosing each urine at its own specific MSL, a “standard” sodium oxalate challenge was also administered to aliquots of each urine. This load was equivalent to the MSL of the control specimens of the particular urine under investigation and was maintained throughout the 12 day experimental period. By administering a fixed NaOx load, comparisons of crystallization rates in different samples could be made. Any trends observed in this approach can be directly attributed to the ascorbic acid regimen. However, in this study no trends in crystallization rates were identified (Tables 6.15 and 6.16). Thus, it can be deduced that, in general, ingestion of ascorbic acid does not affect the rate of *in vitro* calcium oxalate crystallization. However, different individuals may respond differently to the ingestion of ascorbic acid, but this would be dependent on that individual’s renal handling capacity for the vitamin.

7.6 Sedimentation of Calcium Oxalate Crystals in Urine

The time course plot (Figure 6.10) obtained experimentally in this study for the supersaturated urine, is similar to that reported by Hess *et al.* [26] (Figure 3.5). During slow stirring (500 rpm), the number of particles forced downward by gravity equals the number of particles forced upwards by the stirrer [26]. Despite this, the graph reported by Hess *et al.* [26] showed an initial instability for 3 minutes before reaching equilibrium, after which the stirring was stopped and the calcium oxalate sedimentation rate measured. Figure 6.9 does not clearly show the initial period of instability. The period of equilibrium is not as noticeable as in the reported work [26]. This can be attributed to the fact that supersaturation levels were attained by dosing urine at 0.2 M sodium oxalate and monitoring the crystal growth and aggregation by turbidimetry. Unfortunately, the available spectrophotometer was limiting since facilities to stir the urine were unavailable, and hence this method could not be used to detect supersaturation. Numerous attempts to measure the sedimentation rate by turbidimetry were made, but without success. Hence an alternative detection method (spectrophotometry) was used. This necessitated the transfer of urine from one cuvette to another during the period of equilibrium, with the result that this period is not clearly

noticeable in Figure 6.9. Absorbance measurement at time zero is seen to be similar to the measurement at a time of 2 minutes. Thereafter the decrease in absorbance observed for a period of 20 minutes is linear. The slope decreases and tapers off with time. Despite that the linear decrease reported by Hess *et al.* [26] is over a very much shorter time period, before the graph begins to taper off, the trend observed in this study is the same as that observed in the slurry investigation.

Experimentation was carried out using two different urines from the same individual. The results must be regarded as preliminary only, since numerous problems and queries arose. These are addressed in Section 7.6.1. The two urine collections were effected randomly. As a result, no comparison of the effect of ascorbic acid on urinary crystal sizes, derived from the gradients of the plot, can be shown. (The slopes obtained in these urine samples are $0.274 \times 10^{-2} \text{ s}^{-1}$ ($r = 0.9914$) and $4.80 \times 10^{-2} \text{ s}^{-1}$ ($r = 0.9910$), Table 6.17).

7.6.1 Problems Associated with the Experiment

It is vitally important that the urine is stirred at 500 rpm [26] in order to maintain a homogenous solution. A stirring rate of less than 500 rpm does not allow for all the particles to be sufficiently suspended in solution, with the result that there may be a premature sedimenting out of calcium oxalate crystals. The transfer of the urine from the HACH RATIO screw-cap glass cuvette to the LKB NOVOSPEC spectrophotometer tube caused agitation in the solution, thereby destroying its homogeneity and creating the possibility of crystal sedimentation. In this study, adequate facilities to measure and maintain the stirring rate at 500 rpm were not available.

Temperature control is also vitally important in the determination of particle size by means of sedimentation. It is necessary to incubate the system continuously at 37 °C since a change in temperature of 1 °C causes a decrease in reproducibility (private consultation with B. Hess, 1994). In this study, temperature could not be adequately controlled since suitable facilities to accurately incubate the experimental system were unavailable.

Furthermore, the presence of dust in the atmosphere and on glassware is sufficient to affect the reproducibility of the solution and hence the transfer of analyte from one cuvette to another during the analysis would have caused errors in the results obtained.

Stirring results in homogeneity being established, i.e. in the number of particles in the solution forced down due to gravity is equivalent to the number of particles forced up by the stirrer. However Hess *et al.* [26] indicate that despite this, a period of instability occurs until stabilisation is established. Since stirring is not continuous in the present study, due to the transfer of solution from the turbidimeter to the spectrophotometer, it is not possible to determine whether there was a decrease in absorbance initially and when a stable value was obtained. This would increase the error of the system. Thus, without stirring and constant temperature facilities in the spectrophotometer a high possibility of error exists. It is also necessary to run the samples at least in duplicate. Hence, a spectrophotometer which can analyse more than one sample concurrently is essential. Since this facility was not available, it was not possible to perform more than one analysis at a time.

Preliminary data from the analysis is shown in Section 6.5, but the results, although showing literature agreement with those of Hess *et al.* [26], should be regarded as preliminary only. Clearly, this method has potential. Time did not allow the above problems to be resolved. However, future studies involving the role of ascorbic acid in *in vitro* crystallization should incorporate this approach.

7.7 Ascorbic Acid Analysis

Ascorbic acid analysis was carried out on three separate samples collected at pre-determined voidings during each 24 hour collection period for the investigation period for each participant.

The data are characterised by three outliers with the result that the distribution is somewhat skewed. When these outliers are omitted, the estimate of the error variance is reduced by 28 % in magnitude. However, the nature of this study involves a diverse range of factors which may influence the results and thus cause what may appear to be outlying values.

Therefore, these values should not be omitted since they depend on the time of urine voiding, the amount of fluid consumed by the individual and whether or not this has caused diuresis which may result in varied concentrations of ascorbic acid being excreted at different times of the day. A high concentration of ascorbic acid may have been excreted in the voiding immediately prior to analysis resulting in a lower excretion of ascorbic acid in the subsequent voiding. Alternatively, a high ascorbic acid concentration may be excreted if the urine has been retained for a long period in the bladder with the result that all excess ascorbic acid is passed in a single sample. Due to the nature of the collections, outliers were not omitted from the analysis.

7.7.1 Is there a significant change in ascorbic acid excretion after commencement of megadose ingestion of ascorbic acid ?

A significant increase in ascorbic acid excretion relative to control values is observed immediately after commencement of ascorbic acid ingestion (samples 1 and 2 vs sample 3, Table 6.20, Figure 6.11). Statistical analysis was not performed with these results, as the significant increase in ascorbic acid excretion is obvious.

The increase in ascorbic acid excretion as the protocol proceeds is not surprising. The body absorbs the ascorbic acid until the metabolic pool is saturated [8, 27], after which excess ascorbic acid is excreted in the urine. A megadose of ascorbic acid will thus be expected to cause a significant increase in the ascorbic acid excreted in the urine.

7.7.2 Is there a significant change in ascorbic acid excretion during the protocol itself, i.e. as the ingestion of ascorbic acid proceeds ?

Table 6.20 show that there is a significant increase in ascorbic acid excretion as the protocol begins but this appears to level off as the protocol proceeds (Figure 6.10). This is easily explained in terms of the results found by other researchers. The body absorbs ascorbic

acid at a maximal rate of $2.16 \text{ cm}^3 \cdot \text{min}^{-1}$, so that when the vitamin is presented to the tubules at a rate greater than this, all excess ascorbic acid is excreted in the urine [28]. As the ascorbic acid dose increases so the absorption decreases [8, 9, 16] and the process eventually attains a level of supersaturation [7, 12]. The overall result is that an increase in ascorbic acid intake results in an increase in the amount which is excreted unmetabolised in the urine [9]. Therefore, a significant and constant increase in the ascorbic acid excretion during the period of ingestion is not expected, since the body pool would have been saturated resulting in consistently high amounts being excreted in the urine. The plateau effect observed in the present study is consistent with the results of Schmidt *et al.* [17] who reported a similar effect as the body approaches ascorbic acid saturation. It thus appears that although ascorbic acid ingestion continues, the body probably only absorbs what it requires while the remainder is excreted.

7.7.3 Is there a significant change in ascorbic acid excretion immediately post cessation of the protocol ?

Table 6.20 show that there is a significant decrease in the ascorbic acid excreted in the urine during the 24 hours immediately post cessation of ascorbic acid ingestion (sample 6 vs sample 3, 4 and 5) This is not surprising since the body metabolic pool would still be saturated with ascorbic acid and since no further ingestion occurs, there would be less excreted in the urine. From Figure 6.10, it is seen that the ascorbic acid excretion during the 24 hour period immediately post cessation of ascorbic acid ingestion (sample 6) is raised relative to the ascorbic acid excreted during the control collections (samples 1 and 2). This is to be expected, since the body would have been saturated with ascorbic acid for a period of 5 days. Some of this is probably retained and excreted during the day following cessation of the protocol. Thus despite no more ascorbic acid being ingested, there is a “carry-over” from the saturation period, resulting in slightly raised levels of excretion relative to excretion prior to ascorbic acid ingestion.

7.7.4 *Is there a significant change in the urinary excretion of ascorbic acid during the 5 day period post cessation of ascorbic acid ingestion ?*

A continued decreasing trend in urinary ascorbic acid excretion is observed in the 5 day period post ascorbic acid ingestion (samples 6, 7 and 8; Table 6.20, Figure 6.10). The post ingestion period allows the body to rid itself of excess ascorbic acid. As a result, the levels of excretion three days after the cessation of ingestion are still slightly higher than the control values. By the 5th day post cessation of ingestion, the urinary ascorbic acid excretion level has returned to that of the control samples, indicating that a minimum period of about 5 days is required in order for the body to attain normal levels after a period of megadose ingestion of ascorbic acid. Despite the massive ingestion of ascorbic acid for an extended period, the carry over effects are minimal and there does not appear to be any long term effects as a direct result of the ascorbic acid ingestion in any of the 10 participants.

7.7.5 *Is there a significant difference in ascorbate excretion between the three voidings collected at specified times ?*

Table 6.21 shows that there is no significant difference in the three voidings which correspond to specific times during the 24 hour collection, except in the case of sample three. In this sample, voidings "y" and "z" have significantly higher values for the ascorbic acid excreted in the urine than voiding "x" ($P_{x,y} = 0.0023$; $P_{x,z} = 0.0001$), while voiding "y" has a significantly lower value than that of voiding "z" ($P_{y,z} = 0.0107$). This trend can be explained as follows. Sample 3 corresponds to the 24 hour period during which ascorbic acid ingestion commenced. Since ingestion occurred in the morning and since sample "x" corresponds to the second voiding of the day, the initial ascorbic acid boost may be absorbed to a large extent, particularly in cases where the participant was ascorbic acid-deficient. Despite absorption, excretion ($287 \mu\text{g}\cdot\text{cm}^{-3}$ per 24 hours) is higher than in the control samples (78 and $44 \mu\text{g}\cdot\text{cm}^{-3}$ per 24 hours). As ingestion proceeds, decreasing quantities are absorbed as saturation

approaches. This would be accompanied by increasing quantities which are excreted [7, 8] as can be seen with the value of $785 \mu\text{g}.\text{cm}^{-3}$ per 24 hours for voiding "y". It is only after a level of supersaturation is reached that all excess ascorbic acid would be excreted in the urine (value of $1201 \mu\text{g}.\text{cm}^{-3}$ per 24 hours in voiding "z").

After sufficient ascorbic acid has been absorbed to saturate the body pool, all excess ascorbic acid is passed in the urine and thus excretion values for the vitamin, whether in the early morning samples or at other times during the day, would remain raised relative to control values but would not increase relative to each other. The results of the present study therefore suggest that saturation is achieved during the first 24 hours of ascorbic acid ingestion, after which the amount excreted remains constant until ingestion ceases.

Table 6.19 shows the results obtained for ascorbic acid when determined by the manual titration method as well as the flow-injection method developed in the present study. The comparison of the two methods is discussed comprehensively in Chapter 4.

7.7.6 *Summary of the overall trends of ascorbic acid excretion.*

Ascorbic acid excretion increased steadily during the 24 hours following commencement of ingestion. Thereafter, a plateau effect was observed i.e. the amount of ascorbic acid excreted remained high, but constant. It is suggested that initially a large percentage of the ingested vitamin is absorbed which manifests itself by relatively low excretion values. However, it is further suggested that as the body pool of ascorbic acid becomes saturated, less is absorbed but more is excreted. After saturation is achieved, excess ascorbic acid is excreted.

7.8 Tiselius Biochemical Risk Index

Table 6.22 shows the mean Tiselius biochemical risk index [29] for the participants in this study. The probability values determined by least squares comparing the trends observed during the experimental period are shown in Table 6.23. The statistical analysis of the

biochemical risk index showed a fairly skew distribution, but a log transformation of the data is very similar to the untransformed data with the result that only the untransformed data was analysed. Figure 6.11 illustrates the trend of the risk index during the course of the study.

7.8.1 Is there a significant change in the risk index after ingestion of ascorbic acid ?

A comparison of the mean of the two control collections and the mean of the risk index values during the five day period of megadose ascorbic acid ingestion indicate that there is an increase in the risk index values (Table 6.22) and that this increase approaches significance ($p = 0.0665$) (Table 6.23). This indicates that prolonged ingestion of ascorbic acid tends to increase the Tiselius biochemical risk index and hence there is a possible increase in the stone forming potential of the urine (Figure 6.11).

7.8.2 Is there a significant change in the risk index during the protocol itself, i.e. as the ingestion of ascorbic acid proceeds ?

No significant difference in the risk index occurred during the ascorbic acid ingestion period ($p = 0.2842$, Table 6.23). This indicates that although the initial ingestion of ascorbic acid may cause a marginal increase in the stone forming potential of the urine, the continued megadose ingestion of ascorbic acid does not result in a continued increase in the Tiselius biochemical risk index.

7.8.3 Is there a significant change in the risk index immediately post cessation of the protocol ?

The mean value obtained for the risk index for the 10 participants over the period of ascorbic acid ingestion was compared to the mean value for the sample collection on the day

immediately post ascorbic acid cessation (samples 3, 4 and 5 vs sample 6, Table 6.23). A significant change in the risk index values is not observed ($p = 0.4833$), despite cessation of the megadose ingestion.

7.8.4 Is there a significant change in the risk index during the 5 day period following cessation of ascorbic acid ingestion ?

There is no significant change in the day to day risk index values during the five day post cessation period ($p = 0.9426$, Samples 6, 7 and 8, Table 6.23). Thus the risk index values did not decrease significantly after cessation of ascorbic acid ingestion and thus the stone forming potential of the urine after the ingestion of the ascorbic acid remained slightly raised.

7.8.5 Is there a difference between pre-protocol and post-protocol values ?

Tiselius risk index values before the commencement of the protocol are not significantly different to values after the protocol had ended ($p = 0.5748$, Table 6.23). There are thus no long term or delayed effects on this parameter due to ascorbic acid ingestion.

7.8.6 What are the overall trends of and effects on the Tiselius biochemical risk index ?

There is a marginal increase in the risk index at the commencement of ascorbic acid ingestion. This increase in the risk index approaches significance, suggesting that the ingestion of ascorbic acid may increase the stone forming potential of the urine. The continued ingestion of ascorbic acid does not cause a further increase in the risk index values. Therefore prolonged ingestion of ascorbic acid does not increase the risk of stone formation significantly.

7.9 Calcium Oxalate Relative Supersaturation

No significant changes occur in the calcium oxalate relative supersaturation of the urine in the period prior to, during or after ascorbic acid ingestion (Table 6.25). It can thus be concluded that prolonged ingestion of megadose quantities of ascorbic acid does not increase the risk of stone formation.

7.10 Comparison of the Special Case with Participants of the Main Study

It is noteworthy that a difference between the case study and the other 10 participants occurred in that *in vitro* conversion of ascorbic acid to oxalic acid did not occur in the urine of the former but did occur in those of the latter. Further investigation with respect to the renal handling of ascorbic acid by stone formers should address this question.

Another intriguing difference exists between the results obtained for the special case (Chapter 5) and those obtained for the 10 participants in the present investigation. The special case urinary oxalate excretion increased during ascorbic acid ingestion (Table 5.4) while no such increase occurred in the other participants of this investigation. This suggests that *in vivo* conversion of ascorbic acid to oxalic acid occurred in the special case. This might be a consequence of a much larger ascorbic acid load administered to this individual. Alternatively, this important difference might support the contention that the development of crystalluria and haematuria after ascorbic acid ingestion are associated with an impaired renal handling mechanism.

7.11 Concluding Comments

In concluding this thesis, it is appropriate to assess to what extent the objectives of the study, outlined in Section 1.5, have been achieved. A new flow-injection analysis method for the determination of ascorbic acid in human urine has been developed, tested and successfully implemented. The method has been shown to be superior to the manual titration method in terms of precision, economy of solutions and time and has the potential to be fully automated for the rapid routine analysis of ascorbic acid.

In this study, the need for EDTA (or other) preservatives to prevent *in vitro* conversion of ascorbic acid to oxalic acid has been clearly demonstrated. As a result, reliable, reproducible oxalate excretion values were obtained, lending confidence to the credibility of the interpretations on which they were based.

The main objective of the project was to investigate the effect of megadose ingestion of ascorbic acid on several physico-chemical risk factors associated with calcium oxalate kidney stone formation. This has been achieved. As such, the study is original as no other investigation has singly addressed a multitude of risk factors. Rather, studies have been confined to the effects of ascorbic acid ingestion on urinary oxalate in the main. Therefore, the present study represents the first comprehensive investigation of the effects of ascorbic acid ingestion on stone formation risk factors. As far as the results themselves are concerned, these have shown that although there is no increase in the risk of stone formation due to megadose ingestion of ascorbic acid, some individuals (as demonstrated in the special case study), may exhibit adverse effects. This is probably quite rare, with an incidence of 1 in 11 in the present study. Individuals taking ascorbic acid should be aware of the potential dangers and should be wary of any increase in urine cloudiness, which would signify an increase in crystalluria. Perhaps individuals should begin with a low ascorbic acid load and gradually increase the intake to higher levels, to avoid the sudden formation of stones in instances where defective renal handling might result in a pre-disposition to this condition.

7.12 References

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Appendix A

Presentations

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Publications

Kroon, B.L., and Rodgers, A.L., "*Haematuria After Megadose Ingestion Of Vitamin C : A Case Study*" in *Urolithiasis : Consensus and Controversies*, (Eds. Rao, P.N., Kavanagh, J.P., and Tiselius, H-G.), 1994.

[†] The author, Bronwyn Leigh Auer (née Kroon), presented this work prior to her marriage in December 1994.

Appendix B

Graduation of the Jackson Candle Turbidimeter

Table B.1 *Calibration of the glass tube (Jackson Turbidity Units - JTU) which is essential in the structure of the Jackson Candle Turbidimeter.*

| Light Path (cm) | JTU | Light Path (cm) | JTU | Light Path (cm) | JTU |
|-----------------|------|-----------------|-----|-----------------|-----|
| 2.3 | 1000 | 7.0 | 310 | 16.6 | 130 |
| 2.6 | 900 | 7.3 | 300 | 18.0 | 120 |
| 2.9 | 800 | 7.5 | 290 | 19.6 | 110 |
| 3.2 | 700 | 7.8 | 280 | 21.5 | 100 |
| 3.5 | 650 | 8.1 | 270 | 22.6 | 95 |
| 3.8 | 600 | 8.4 | 260 | 23.8 | 90 |
| 4.1 | 550 | 8.7 | 250 | 25.1 | 85 |
| 4.5 | 500 | 9.1 | 240 | 26.5 | 80 |
| 4.9 | 450 | 9.5 | 230 | 28.1 | 75 |
| 5.5 | 400 | 9.9 | 220 | 29.8 | 70 |
| 5.6 | 390 | 10.3 | 210 | 31.8 | 65 |
| 5.8 | 380 | 10.8 | 200 | 34.1 | 60 |
| 5.9 | 370 | 11.4 | 190 | 36.7 | 55 |
| 6.1 | 360 | 12.0 | 180 | 39.8 | 50 |
| 6.3 | 350 | 12.7 | 170 | 43.5 | 45 |
| 6.4 | 340 | 13.5 | 160 | 48.1 | 40 |
| 6.6 | 330 | 14.4 | 150 | 54.0 | 35 |
| 6.8 | 320 | 15.4 | 140 | 61.8 | 30 |

Appendix C

Table C.1 *Preparation of sodium oxalate dosing concentrations.*

| Dosing Conc. No. | Desired NaOx Conc. | Vol. NaOx Stock | Vol. water added |
|------------------|--------------------|-----------------|------------------|
| 1 | 0.01 | 0.50 | 9.50 |
| 2 | 0.02 | 1.00 | 9.00 |
| 3 | 0.03 | 1.50 | 8.50 |
| 4 | 0.04 | 2.00 | 8.00 |
| 5 | 0.05 | 2.50 | 7.50 |
| 6 | 0.06 | 3.00 | 7.00 |
| 7 | 0.07 | 3.50 | 6.50 |
| 8 | 0.08 | 4.00 | 6.00 |
| 9 | 0.09 | 4.50 | 5.50 |
| 10 | 0.10 | 5.00 | 5.00 |
| 11 | 0.11 | 5.50 | 4.50 |
| 12 | 0.12 | 6.00 | 4.00 |
| 13 | 0.13 | 6.50 | 3.50 |
| 14 | 0.14 | 7.00 | 3.00 |
| 15 | 0.15 | 7.50 | 2.50 |
| 16 | 0.16 | 8.00 | 2.00 |
| 17 | 0.17 | 8.50 | 1.50 |
| 18 | 0.18 | 9.00 | 1.00 |
| 19 | 0.19 | 9.50 | 0.50 |
| 20 | 0.20 | 10.0 | 0.00 |

Appendix D

Table D.1 *Urinary calcium excretion (mmol/24 hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 2.60 | 2.32 | 6.75 | 5.18 | 1.05 | 0.33 | 0.55 | 3.03 | 4.75 | 0.75 |
| 2 | 2.46 | 4.62 | 3.68 | 5.42 | 1.83 | 0.60 | 1.34 | 1.95 | 1.05 | 0.66 |
| 3 | 2.84 | 4.00 | 4.41 | 7.36 | 2.84 | 1.16 | 1.07 | 1.77 | 3.06 | 0.48 |
| 4 | 2.46 | 5.28 | 3.62 | 4.60 | 1.64 | 0.73 | 1.90 | 2.20 | 3.66 | 1.16 |
| 5 | 2.47 | 5.70 | 4.91 | 3.32 | 2.05 | 0.65 | 1.71 | 2.10 | 4.18 | 0.84 |
| 6 | 3.76 | 6.33 | 2.73 | 13.2 | 1.25 | 0.52 | 1.21 | 1.79 | 5.10 | 0.33 |
| 7 | 1.70 | 2.93 | 5.55 | 4.04 | 1.28 | 0.53 | 0.97 | 0.99 | 2.61 | 0.98 |
| 8 | 2.17 | 2.47 | 3.95 | 7.11 | 1.28 | 0.55 | - | 1.72 | 3.42 | 0.52 |

Table D.2 *Urinary magnesium excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 4.21 | 3.67 | 4.77 | 4.08 | 3.20 | 2.20 | 1.31 | 1.16 | 5.31 | 1.69 |
| 2 | 3.26 | 4.68 | 3.05 | 3.20 | 5.73 | 2.49 | 2.57 | 1.99 | 2.03 | 1.41 |
| 3 | 4.46 | 5.90 | 5.45 | 5.72 | 6.65 | 3.54 | 2.45 | 3.47 | 3.61 | 1.74 |
| 4 | 3.18 | 6.81 | 6.77 | 5.84 | 3.98 | 2.82 | 2.42 | 2.49 | 3.96 | 2.63 |
| 5 | 3.05 | 7.05 | 3.59 | 2.51 | 4.10 | 3.37 | 2.45 | 2.60 | 4.03 | 3.54 |
| 6 | 3.51 | 6.84 | 3.07 | 7.40 | 2.96 | 2.40 | 1.84 | 2.13 | 4.85 | 0.96 |
| 7 | 2.28 | 3.95 | 3.49 | 3.05 | 3.47 | 2.68 | 2.65 | 4.09 | 2.80 | 1.67 |
| 8 | 1.92 | 3.38 | 4.32 | 3.62 | 3.16 | 2.74 | - | 4.22 | 2.99 | 1.60 |

Table D.3 *Urinary sodium excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|
| 1 | 117.6 | 136.2 | 95.8 | 109.1 | 66.7 | 33.4 | 58.9 | 90.6 | 250.6 | 22.2 |
| 2 | 114.7 | 209.5 | 64.0 | 49.8 | 89.1 | 40.1 | 133.4 | 100.8 | 136.7 | 17.8 |
| 3 | 138.5 | 139.2 | 70.9 | 120.9 | 105.0 | 61.5 | 76.7 | 65.8 | 260.9 | 48.0 |
| 4 | 102.3 | 129.4 | 70.9 | 158.2 | 15.5 | 21.5 | 73.6 | 97.2 | 105.3 | 89.1 |
| 5 | 110.6 | 107.6 | 143.6 | 48.7 | 67.8 | 54.1 | 48.3 | 113.4 | 169.4 | 84.0 |
| 6 | 129.1 | 154.8 | 70.8 | 230.6 | 38.1 | 40.9 | 112.2 | 65.5 | 289.8 | 21.1 |
| 7 | 119.1 | 62.6 | 75.2 | 101.9 | 22.2 | 15.1 | 80.4 | 28.2 | 59.9 | 54.2 |
| 8 | 127.3 | 56.4 | 181.2 | 155.1 | 60.1 | 113.1 | - | 126.3 | 132.9 | 56.4 |

Table D.4 *Urinary potassium excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|-------|-------|-------|------|------|------|------|-------|------|
| 1 | 45.6 | 43.2 | 146.6 | 71.9 | 50.1 | 31.5 | 20.5 | 97.0 | 91.6 | 10.6 |
| 2 | 44.7 | 73.6 | 44.5 | 36.9 | 51.5 | 44.6 | 51.8 | 69.0 | 76.2 | 25.5 |
| 3 | 73.2 | 102.4 | 123.4 | 58.9 | 61.2 | 39.9 | 15.1 | 87.4 | 79.3 | 16.3 |
| 4 | 67.0 | 55.4 | 134.5 | 111.2 | 22.2 | 47.5 | 20.9 | 29.5 | 109.5 | 12.8 |
| 5 | 53.5 | 84.5 | 18.4 | 40.9 | 49.6 | 44.7 | 12.1 | 32.0 | 62.0 | 22.7 |
| 6 | 49.0 | 106.4 | 13.8 | 60.1 | 38.5 | 46.8 | 15.1 | 28.3 | 80.4 | 4.91 |
| 7 | 69.8 | 44.6 | 17.8 | 39.8 | 33.5 | 32.2 | 4.90 | 14.9 | 53.4 | 22.9 |
| 8 | 64.9 | 37.9 | 47.7 | 43.5 | 39.3 | 73.2 | - | 88.9 | 84.0 | 22.0 |

Table D.5 *Urinary citrate excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 2.63 | 3.20 | 3.08 | 3.09 | 2.11 | 1.12 | 1.20 | 1.67 | 3.12 | 0.44 |
| 2 | 3.16 | 3.67 | 2.68 | 1.95 | 2.97 | 1.33 | 1.34 | 1.56 | 1.98 | 0.93 |
| 3 | 2.55 | 2.62 | 2.68 | 2.47 | 2.90 | 1.49 | 1.25 | 1.34 | 2.36 | 1.42 |
| 4 | 1.93 | 5.37 | 3.58 | 1.99 | 2.00 | 1.63 | 1.30 | 1.03 | 2.29 | 1.11 |
| 5 | 1.48 | 3.16 | 2.38 | 2.41 | 2.03 | 2.37 | 1.04 | 1.24 | 2.50 | 1.97 |
| 6 | 1.90 | 2.82 | 1.27 | 2.77 | 2.25 | 1.64 | 0.80 | 1.24 | 3.76 | 0.69 |
| 7 | 2.97 | 2.90 | 1.91 | 2.72 | 1.79 | 1.37 | 1.28 | 0.93 | 2.33 | 2.32 |
| 8 | 1.94 | 1.86 | 4.15 | 3.27 | 1.78 | 2.55 | - | 1.27 | 2.72 | 0.57 |

Table D.6 *Urinary pH for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 5.35 | 6.55 | 6.50 | 6.55 | 6.05 | 5.85 | 6.15 | 6.65 | 6.55 | 4.30 |
| 2 | 6.00 | 6.20 | 6.35 | 5.45 | 6.35 | 6.15 | 6.55 | 6.85 | 6.95 | 5.50 |
| 3 | 5.20 | 5.95 | 6.10 | 5.90 | 6.25 | 6.10 | 6.20 | 6.35 | 6.85 | 5.85 |
| 4 | 5.60 | 6.00 | 5.95 | 6.20 | 5.50 | 6.00 | 6.15 | 6.25 | 6.55 | 5.85 |
| 5 | 5.20 | 5.90 | 5.65 | 6.40 | 6.45 | 6.45 | 6.05 | 6.10 | 6.20 | 5.85 |
| 6 | 5.55 | 6.35 | 5.55 | 6.10 | 5.65 | 6.05 | 6.40 | 6.45 | 6.70 | 6.35 |
| 7 | 6.05 | 6.15 | 5.30 | 5.60 | 6.30 | 6.25 | 6.35 | 6.05 | 6.65 | 6.55 |
| 8 | 5.30 | 6.05 | 6.70 | 6.05 | 5.95 | 7.00 | - | 5.95 | 6.55 | 6.10 |

Table D.7 *Urinary volume (cm³) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 1260 | 2320 | 1160 | 1420 | 920 | 1385 | 2120 | 980 | 2560 | 440 |
| 2 | 1860 | 2140 | 1840 | 975 | 1560 | 900 | 2375 | 840 | 1330 | 1240 |
| 3 | 3140 | 2000 | 2410 | 2350 | 1045 | 895 | 2035 | 1260 | 2275 | 1210 |
| 4 | 1920 | 1985 | 2410 | 2780 | 1050 | 990 | 2160 | 735 | 1420 | 2420 |
| 5 | 1660 | 1905 | 1440 | 2170 | 775 | 950 | 1800 | 1360 | 1815 | 1985 |
| 6 | 1780 | 1815 | 1545 | 1040 | 735 | 950 | 2150 | 1095 | 2080 | 1140 |
| 7 | 1810 | 1600 | 1995 | 1860 | 1040 | 1260 | 1895 | 1075 | 1835 | 1830 |
| 8 | 1440 | 1235 | 2170 | 2210 | 570 | 950 | - | 1400 | 1760 | 1760 |

Table D.8 *Urinary urate excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 3.30 | 3.48 | 4.70 | 3.90 | 2.59 | 2.24 | 2.40 | 2.50 | 5.68 | 0.66 |
| 2 | 4.24 | 3.50 | 4.44 | 3.00 | 3.65 | 2.38 | 4.10 | 2.40 | 2.87 | 1.27 |
| 3 | * | * | * | * | * | * | * | * | * | * |
| 4 | * | * | * | * | * | * | * | * | * | * |
| 5 | * | * | * | * | * | 0.07 | * | * | * | * |
| 6 | 2.03 | 1.85 | * | 1.0 | 0.37 | 0.19 | 1.20 | 0.90 | 2.37 | 0.35 |
| 7 | 4.89 | 3.50 | * | 2.57 | 2.56 | 2.19 | 2.50 | 1.10 | 2.97 | 2.31 |
| 8 | 3.11 | 2.59 | 2.99 | 4.91 | 2.40 | 3.20 | - | 2.40 | 1.88 | 3.90 |

* : urate excretion could not be determined in urines with a high ascorbic acid load due to the interference and masking effects of the vitamin on the determination of urate.

Table D.9 *Urinary creatinine excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|-----|------|------|
| 1 | 14.3 | 15.3 | 12.6 | 14.9 | 10.2 | 9.4 | 10.2 | 9.8 | 19.2 | 5.0 |
| 2 | 16.1 | 14.5 | 13.5 | 13.6 | 14.9 | 11.4 | 11.8 | 9.4 | 11.2 | 6.5 |
| 3 | 16.3 | 14.8 | 12.9 | 15.1 | 13.9 | 11.9 | 12.3 | 9.7 | 15.2 | 6.9 |
| 4 | 14.0 | 17.1 | 12.9 | 16.9 | 12.0 | 11.9 | 7.0 | 9.3 | 14.8 | 7.5 |
| 5 | 13.9 | 13.4 | 13.0 | 17.7 | 11.2 | 11.8 | 10.3 | 8.9 | 16.2 | 8.5 |
| 6 | 15.1 | 14.9 | 9.3 | 16.0 | 12.9 | 11.2 | 8.3 | 7.5 | 15.0 | 2.3 |
| 7 | 17.6 | 11.0 | 14.1 | 15.0 | 12.2 | 10.6 | 11.6 | 6.9 | 15.4 | 8.4 |
| 8 | 13.0 | 9.0 | 14.1 | 16.8 | 10.7 | 12.7 | - | 9.6 | 6.6 | 15.0 |

Table D.10 *Urinary phosphate excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 30.2 | 32.2 | 34.7 | 25.7 | 18.8 | 15.7 | 13.7 | 22.8 | 39.6 | 10.0 |
| 2 | 42.2 | 31.0 | 39.0 | 20.4 | 28.4 | 18.5 | 19.5 | 18.2 | 27.9 | 15.3 |
| 3 | 31.1 | 27.2 | 31.1 | 25.8 | 26.3 | 18.8 | 12.7 | 22.7 | 35.6 | 13.5 |
| 4 | 24.8 | 40.1 | 34.6 | 33.2 | 22.4 | 18.2 | 15.7 | 13.7 | 33.0 | 8.3 |
| 5 | 27.1 | 35.1 | 34.1 | 20.2 | 19.2 | 29.9 | 13.7 | 18.6 | 36.4 | 15.1 |
| 6 | 27.2 | 34.1 | 17.8 | 21.7 | 22.6 | 21.6 | 10.3 | 11.6 | 29.9 | 3.3 |
| 7 | 28.8 | 26.2 | 8.9 | 26.0 | 24.8 | 19.8 | 15.3 | 14.0 | 22.6 | 16.6 |
| 8 | 31.0 | 18.9 | 22.1 | 33.5 | 23.5 | 21.6 | - | 24.9 | 14.1 | 28.1 |

Table D.11 *Urinary chloride excretion (mmol/24hr) for each 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1 | 110 | 195 | 140 | 114 | 196 | 89 | 66 | 100 | 230 | 27 |
| 2 | 162 | 214 | 59 | 70 | 198 | 117 | 140 | 105 | 107 | 29 |
| 3 | 170 | 176 | 87 | 98 | 256 | 147 | 63 | 95 | 189 | 42 |
| 4 | 129 | 119 | 60 | 136 | 97 | 106 | 48 | 87 | 97 | 102 |
| 5 | 144 | 108 | 140 | 154 | 184 | 141 | 54 | 106 | 158 | 81 |
| 6 | 198 | 168 | 77 | 132 | 129 | 168 | 112 | 83 | 154 | 22 |
| 7 | 186 | 72 | 84 | 112 | 105 | 64 | 63 | 41 | 44 | 122 |
| 8 | 166 | 80 | 156 | 197 | 104 | 202 | - | 116 | 55 | 112 |

Table D.12 *Urinary oxalate excretion (mmol/24hr) for each "bulk" 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 0.32 | 0.33 | 0.21 | 0.21 | 0.26 | 0.14 | 0.10 | 0.17 | 0.19 | 0.05 |
| 2 | 0.23 | 0.21 | 0.20 | 0.12 | 0.22 | 0.19 | 0.16 | 0.20 | 0.15 | 0.14 |
| 3 | 0.26 | 0.14 | 0.12 | 0.34 | 0.30 | 0.15 | 0.21 | 0.30 | 0.24 | 0.16 |
| 4 | 0.35 | 0.11 | 0.16 | 0.26 | 0.13 | 0.19 | 0.24 | 0.35 | 0.19 | 0.20 |
| 5 | 0.22 | 0.15 | 0.13 | 0.26 | 0.14 | 0.30 | 0.56 | 0.42 | 0.16 | 0.21 |
| 6 | 0.17 | 0.21 | 0.10 | 0.20 | 0.15 | 0.19 | 0.22 | 0.14 | 0.24 | 0.04 |
| 7 | 0.20 | 0.12 | 0.22 | 0.26 | 0.28 | 0.42 | 0.17 | 0.10 | 0.16 | 0.11 |
| 8 | 0.17 | 0.12 | 0.35 | 0.20 | 0.27 | 0.22 | - | 0.19 | 0.13 | 0.10 |

Table D.13 *Urinary oxalate excretion (mmol/24hr) for each “+ EDTA” 24 representative hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 0.30 | 0.12 | 0.09 | 0.13 | 0.11 | 0.11 | 0.06 | 0.14 | 0.48 | 0.03 |
| 2 | 0.16 | 0.10 | 0.12 | 0.11 | 0.14 | 0.13 | 0.17 | 0.16 | 0.24 | 0.14 |
| 3 | 0.30 | 0.18 | 0.09 | 0.17 | 0.16 | 0.13 | 0.14 | 0.20 | 0.38 | 0.08 |
| 4 | 0.32 | 0.10 | 0.09 | 0.18 | 0.11 | 0.13 | 0.16 | 0.21 | 0.29 | 0.17 |
| 5 | 0.19 | 0.14 | 0.17 | 0.18 | 0.09 | 0.13 | 0.28 | 0.26 | 0.20 | 0.07 |
| 6 | 0.16 | 0.11 | 0.06 | 0.10 | 0.09 | 0.11 | 0.22 | 0.07 | 0.41 | 0.04 |
| 7 | 0.14 | 0.10 | 0.19 | 0.21 | 0.16 | 0.15 | 0.12 | 0.19 | 0.14 | 0.08 |
| 8 | 0.13 | 0.10 | 0.22 | 0.18 | 0.08 | 0.10 | - | 0.28 | 0.26 | 0.07 |

Table D.14 *Urinary oxalate excretion (mmol/24hr) for each “-EDTA” representative 24 hour urine sample collected by the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|------|------|
| 1 | 0.27 | 0.22 | 0.17 | 0.22 | 0.20 | 0.23 | 0.12 | 0.21 | 0.30 | 0.04 |
| 2 | 0.26 | 0.14 | 0.21 | 0.13 | 0.14 | 0.15 | 0.16 | 0.18 | 0.27 | 0.26 |
| 3 | 0.35 | 0.17 | 0.11 | 0.23 | 0.29 | 0.21 | 0.15 | 0.26 | 0.29 | 0.14 |
| 4 | 0.43 | 0.10 | 0.15 | 0.24 | 0.17 | 0.22 | 0.34 | 0.32 | 0.31 | 0.23 |
| 5 | 0.22 | 0.18 | 0.29 | 0.30 | 0.20 | 0.24 | 0.60 | 0.48 | 0.26 | 0.15 |
| 6 | 0.18 | 0.35 | 0.10 | 0.17 | 0.21 | 0.20 | 0.23 | 0.25 | 0.26 | 0.05 |
| 7 | 0.16 | 0.15 | 0.32 | 0.28 | 0.42 | 0.36 | 0.18 | 0.11 | 0.17 | 0.16 |
| 8 | 0.23 | 0.22 | 0.32 | 0.32 | 0.21 | 0.21 | - | 0.17 | 0.18 | 0.13 |

Table D.15 *Urinary ascorbic acid excretion ($\mu\text{g}\cdot\text{cm}^{-3}$) for voiding "x" collected by each of the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-----|------|-----|------|------|------|------|------|------|-----|
| 1 | 33 | 13 | 21 | 47 | 12 | - | 18 | 37 | - | 239 |
| 2 | 9 | 55 | 14 | 59 | 37 | 13 | 12 | 16 | 28 | 192 |
| 3 | 249 | 39 | 37 | 15 | 1127 | 937 | 31 | 26 | 145 | 263 |
| 4 | 201 | 874 | 835 | 1365 | 1490 | 1396 | 2018 | 1138 | 929 | 89 |
| 5 | 276 | 1865 | 977 | 727 | 1306 | - | 422 | 1162 | 755 | 72 |
| 6 | 308 | 580 | 528 | 822 | - | 627 | 552 | 473 | 1057 | 35 |
| 7 | 25 | 61 | 50 | 279 | 284 | 173 | 6 | 489 | 29 | 11 |
| 8 | 14 | 31 | 70 | 24 | 46 | 135 | - | 55 | 29 | 21 |

Table D.16 *Urinary ascorbic acid excretion ($\mu\text{g}\cdot\text{cm}^{-3}$) for voiding "y" collected by each of the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|-----|------|------|------|------|------|-----|------|-----|-----|
| 1 | 59 | 4 | 16 | 49 | 8 | - | - | 20 | - | 73 |
| 2 | 11 | 14 | 18 | 56 | 8 | 21 | 41 | 46 | 20 | 209 |
| 3 | 414 | 1542 | 682 | 496 | 2313 | 1240 | 20 | 577 | 264 | 306 |
| 4 | 581 | 219 | 1217 | 1114 | 1240 | 1866 | 81 | 1388 | 661 | 87 |
| 5 | 905 | 181 | 1096 | 699 | 1168 | 1220 | 349 | 982 | 965 | 58 |
| 6 | 67 | 474 | 112 | 516 | - | - | 26 | 141 | 725 | 70 |
| 7 | 77 | 24 | 82 | 307 | 21 | - | 5 | 209 | 45 | 17 |
| 8 | 28 | 6 | 93 | 152 | 12 | - | - | 22 | 45 | 18 |

Table D.17 *Urinary ascorbic acid excretion ($\mu\text{g}\cdot\text{cm}^{-3}$) for voiding "z" collected by each of the 10 participants.*

| # | PT | ES | PR | SH | MG | GV | BM | CW | RG | AM |
|---|------|------|------|------|------|------|------|------|-----|------|
| 1 | 19 | 81 | 18 | 85 | 40 | 19 | 26 | 20 | - | 87 |
| 2 | 12 | 67 | 4 | 25 | 14 | 16 | 22 | 30 | 23 | 44 |
| 3 | 278 | 1461 | 523 | 1220 | 1521 | 1283 | 1510 | 1982 | 929 | 1306 |
| 4 | 1257 | 1121 | 1003 | 1189 | 790 | 184 | 88 | 2643 | 607 | 463 |
| 5 | 962 | 1095 | 1181 | 1243 | 2675 | 1062 | 624 | 1194 | 804 | 478 |
| 6 | 97 | 62 | 160 | 459 | 190 | 531 | 183 | 226 | 97 | 8 |
| 7 | 22 | 32 | 600 | 33 | 16 | 6 | 12 | 155 | 25 | 20 |
| 8 | 19 | 25 | 195 | 15 | 13 | 66 | - | 22 | 19 | 9 |

Appendix E

Table E.1 *Summary of the manifold details used in the determination of ascorbic acid using 2,6 DCPIP in a rFIA manifold.*

| Parameter | Description |
|-------------------------|---|
| Peristaltic Pump | Gilson Minipuls 2; PVC calibrated pump tubes (yellow tags); flow rates were calibrated gravimetrically |
| Injection Valve | Rheodyne Type 5020 teflon rotary injection valve. |
| Manifold Tubing | 0.5 mm and 0.8 mm inner diameter tubing was used throughout. |
| Reaction Coil | A 100 cm length of tubing (0.5 mm i.d.) was tightly coiled around a teflon cylinder (i.d. = 15 mm) and firmly mounted. |
| Detector | An LKB Novaspec spectrophotometer was employed in time-base mode, the output analogue port was connected to an SP4290 integrator. |
| Flow Cell | Florescence flow through cell, U-shaped of constant pathlength 0.205 cm. |

Appendix F

Figure F.1 *The difference of the FIA - manual titration methods plotted against the average of the two methods. Confidence limits are drawn at 95 % confidence.*

Difference (flow injection - manual titration) methods plotted against average

